Studies on the in vitro regeneration and micropropagation of Cucumis sativus L. (cucumber)

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STUDIES ON THE IN VITRO REGENERATION AND MICROPROPAGATION OF CUCUMIS SATIVUS L. (CUCUMBER).

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To my wife

Esi

and our sons

Hamed, Hosam and Ehsan

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ABSTRACT.

A protocol was developed for the continuous clonal propagation of cucumber in vitro using three cultivars. The presence of either a cytokinin or of an auxin was found to be necessary and sufficient to ensure continuous proliferation. Concentrations of 0.4 μM for BA and of 0.3 μM for NAA were found to give most satisfactory results and were found to be superior to combined applications of the two hormones. An unusual feature of these experiments was premature flowering; this was cultivar-dependent and affected cultures grown on NAA more than those on BA. Such transition to the reproductive growth phase could be inhibited by growing the cultures at 30°C.

A suitable protocol was also established for the low labour-input maintenance of cucumber cultures. Temperatures below 15°C were found to be lethal. The survival rate of cultures kept under osmotic stress [3% mannitol] at 20°C for 3 months without transfer was 100%; transition to flowering was minimal.

Immature inflorescence cultures were used to assess the effect of IAA, 2-chloroethylphosphonic acid and of photoperiod on sex expression in vitro, using four genotypes. IAA, in the concentration range tested, left the sex expression of the inflorescences largely unaffected [near 100% male flowers]. 2-chloroethylphosphonic acid induced the formation of a small percentage of female flowers. A further experiment, using one of the previously tested genotypes and two others has shown that it was the photoperiod, together with the genotype, that determined sex expression in these experiments: in two out of three genotypes tested, sex expression could be manipulated solely by changing the photoperiod.

A protocol was also devised for the adventitious regeneration of shoots from unexpanded leaves; 0.25 μM BA was found to be the optimal hormone concentration. Using this procedure, 747 R_1 plantlets and 115 R_2 plantlets were regenerated from a genotype heterozygous for the bitterness gene (Nbnb). No plantlets with the non-bitter phenotype could be detected among the regenerants; the somaclonal variation rate was too low to produce mutants of a single gene with a high enough frequency.

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LIST OF ABBREVIATIONS

BA	6-benzyladenine
2,4-D	2,4-dichlorophenoxyacetic acid
IAA	indole -3- acetic acid
MS	Murashige & Skoog (1962) medium
NAA	naphthaleneacetic acid
KIN	kinetin
PGR	Plant growth regulators
CM	coconut milk
GA_3	gibberellic acid
DMSO	dimethylsulphoxide
TBA	tertiary butyl alcohol
cv.	cultivar
cvs.	cultivars
L/D	a length to diameter ratio
PE	plating efficiency
2ip	(2-Isopentenyl) adenine

1.Cucumber ; biology and economics.

a.Taxonomy and morphology of cucumber (Cucumis sativus L.)

There are about 110 genera and 640 species in the Cucurbitaceae family. The cultivated species of this family belong to the genera Benincasa, Citrullus, Cucumis, Cucurbita, Langenaria, Luffa, Momordica, Sechium and Trichosanthes (Everett , 1981).

The genus Cucumis consists of about 40 species, but only two of them are commonly cultivated, Cucumis melo, the sweet melon (2n=24), and Cucumis sativus L., the cucumber and gherkin (2n=14)(Bassett, 1986).

The cucumber is one of the most widely grown members of the Cucurbitaceae family . It has been cultivated since very early times, indeed some authorities claim that it was grown three thousand years ago (DE Candolle, 1964; Bassett, 1986). Cucumber is native to Asia or tropical Africa (Rayle et al, 1980; Splittstosser, 1984; Hartmann et al, 1988), although owing to its universal popularity some doubt exists as to the exact country or region of its origin.

The cucumber plant is a prostrate, annual vining plant with stiff hairs or spines on leaves and stems. Unbranched lateral tendrils develop at the leaf axils, and vining begins after two or three true leaves form. Branching also begins at this time. As soon as lateral branches develop, flower clusters appear at the leaf axils (Peirce, 1987).

The cucumber plant is monoecious. The first flowers generally staminate, are followed by pistillate flowers, which, when fertilized, develop into fruit (Hartmann, 1988). Many newly

Cucumbers exhibit a fascinating range of floral morphology. Within C. sativus, staminate, pistillate and hermaphroditic flowers occur in various arrangements, yielding several types of sex expression. Furthermore, these types are influenced greatly by environmental conditions, producing a virtually continuous spectrum of sex expression (Lower & Edwards, 1986).

Cucumber is one of the relatively few genetically well characterized higher plant species (Whitaker & Robinson, 1974); the classical paper on the genetics of the cucumber is that by Robinson et al (1976). The phenotypes of sex expression in Cucurbitaceae are summarised in Table 1. Dioecy (male and female flowers on separate individuals) does not occur in this family. Flowers are either male (staminate), female (pistillate) or perfect (both staminate and pistillate); the three types of flowers are known to occur on individual plants in all combinations of the 1, 2 or 3 types.

The genes affecting sex determination in cucumber together with the corresponding phenotypes are shown in Table 1.a.

There is no simple link between genotype and phenotype (Robinson et al, 1976) because of the presence of intensifier genes, such as *In-F*, gene interactions and ubiquitous environmental effects.

A good deal of research has been done on the effect of daylength and temperature on sex expression of cucurbits, especially of cucumber, muskmelon and squash (Nitsch et al, 1952; Fukushima et al, 1968; Masto et al, 1969; Masto et al,

Table 1- .: Phenotypes of sex expression in Cucurbitaceae.

Symbols: ♀ female (pistillate) flowers.

♂ male (staminate) flowers.

⚥ perfect (pistillate and staminate) flowers.

Adopted from Robinson et al,1976.

Types	Flowers born on individual plants
Gynoecious	♀ only
Androecious	♂ only
Hermaphroditic	⚥ only
Monoecious	♀ and ♂
Gynomonoecious	♀ and ⚥
Andromonoecious	♂ and ⚥
Trimonoecious	♂ , ♀ and ⚥

Table 1.d: Genes controlling sex expression in cucumber .
From Robinson et al, 1976.

Preferred symbol	Synonym	Character	Reference
a		androecious. produce primarily staminate flowers if recessive *F*.	Kooistra 1969a
F	(Acr, acr^F, D,st)	Female. high degree of female sex expression; interacts with *a* and *M*; strongly modified by enviroment and genetic backgrounds.	Tkachenko 1935
gy	(*g*)	Gynoecious. Recessive gene for high degree of female sex expression.	Kooistra 1974
In-F	*F*	Intensifier of female sex expression. Increases degree of female sex expression of *F* plants.	Kooistra 1969b.
m	(*a, g,mo*)	andromonoecious. Plants are andromonoecious if m^+; ++ = monoecious, +*F* = gynoecious, *mF* = hermaphroditic.	Rosa 1928 Tkachenko 1935
m-2	(*h*)	andromonoecious-2. Bisexual flowers with normal ovaries.	Kooistra 1974
Tr		Trimonoecious. Producing male, bisexual, and female flowers in this sequence during plant development.	Kooistra 1969c

1969 and Rudich et al, 1976). The findings of Nitsch et al (1952), showed that low temperatures, combined with short day conditions, promote formation of female flowers. conversely, high temperatures with long day conditions were found to promote a tendency towards male flowers in cucumber and squash. However, many exceptions to this rule have been noted later. Some cucumber cultivars, for instance, showed sensitivity to day length, short days enhancing femaleness, while others remained unaffected. Another cultivar displayed a tendency to femaleness under long day conditions (Fukushima et al, 1968; Masto & Fukushima, 1969).

Eisuk et al (1968) determined that for the cucumber variety Higanfishinari 8 hr days and blue light stimulated the formation of male flowers on lower internodes of the stem, while the formation of female flowers was inhibited. Red light resulted in the formation of predominantly vegetative buds.

The response to low temperature is also equivocal. Low temperatures promote femaleness in some cultivars, while they have no effect on the others. Similar behaviour was also found with muskmelon (Rudich & Peles, 1976).

The effect of temperature in the determination of sex expression in watermelon differs from that of cucumber and squash (Nitsch et al, 1952). Whereas night temperature appeared to be the determinig factor in the increase of gynoecious expression of cucumber and squash, it had no influence on sex expression of watermelon. These findings suggest that the day temperature is one of the factors regulating sex expression of the watermelon. Temperatures of 32°C suppressed femaleness and promoted a tendency towards maleness (Rudich & Peles, 1976).

Exogenous hormones can also modify the sexuality of flowers suggesting that hormones mediate genetic and environmental control of sex expression (Metzger, 1987).

In Cucumis, perfect flowers are initiated but one sex organ sometimes fails to develop (Metzger, 1987). Application of plant growth regulators, in particular, auxin and ethylene are known to induce feminization in cucumber and other cucurbits (Galun, 1959; Rudich, 1985). Pistillate flower formation resulted from application of auxin to cucumber plants or floral buds in the bisexual stage (Galun, 1963). Using bioassays, IAA content in apices of gynoecious plants was found to be higher than in monoecious apices (Rudich, 1972). On the other hand , lower auxin levels were found under environmental conditions which promote a femaleness (Saito & Ito, 1964). In other words high production of ethylene and pistillate flower formation were found following application of auxin (Shannon & De La Guardia, 1969). Hence, it has been suggested that ethylene is the major factor responsible for female sex expression, while auxin acts indirectly by stimulating ethylene production (Rudich, 1972, 1985). Inhibition of ethylene biosynthesis by aminothoxyvinylglycine (AVG) or ethylene action by $AgNo_3$ suppressed female sex expression in gynoecious cucumber plants (Atsmon & Tabback, 1979). Inhibitors of GA biosynthesis, on the other hand, caused a tendency towards feminization (Halevy & Rudich, 1967; Pharis & King, 1985).

Endogenous hormone levels are also consistent with their postulated role in sex expression (Metzger, 1987). Shoots of a hermaphroditic line of Cucumis had a higher auxin content than an andromonoecious line (Galun et al, 1965). Endogenous levels of ethylene and GA-like substances were also found to be correlated with sex expression: high levels of ethylene production were associated with plants containing pistillate flowers (Byers & Baker, 1972; Rudich et al, 1972) and staminate plants

contained more GA-like substances than their female counterparts (Rudich et al, 1972b). Treatments with exogenous gibberellin increased maleness in cucumbers (Peterson & Anhder, 1960) or delayed female flower formation (Bukovac & Withers, 1961).

In total, the results strongly suggest that sex expression in Cucumis and cucurbits is partly regulated by the internal balance of auxins acting through ethylene and GAs.

Neverthless, the hormonal balance is not the sole factor in determining sex expression in Cucumis . In a C. sativus cultivar in which the number of pistillate flowers increased in response to short days , the endogenous levels of GA-like substances were higher and ethylene production lower in short days than long ones, the opposite to what one would predict (Takahashi et al, 1983). This suggests that environmental control of sex expression in this species is not necessarily mediated through a balance of GAs and ethylene.

Cannabis sativa is a dioecious plant in which, like Cucumis, flower primordia are uncommitted at the time of flower initiation. Application of auxins, ethylene, and GAs affect sex expression in similar fashion as in Cucumis (Chailakhyan & Khrianin, 1980; Heslop-Harrison, 1972; Pharis & King, 1985). Growth retardants feminize Cannabis plants, which can be reversed by GA_3. In addition, cytokinins also promote femaleness following experiments in which plants were defoliated or derooted, it was proposed that leaves play an essential role in sex expression in Cannabis by supplying GAs to the flower bud. Male plants tend to have higher levels of GA-like substances as well. An increased cytokinin content was associated with female plants (Chailakhyan & Khrianin, 1980).

Cucumbers are self-compatible but are predominantly cross-pollinated. Pollination is mainly done by bees when the plants are grown as a field crop. Cucumber is not cross-compatible with other genera of cucurbitaceae family (George, 1985).

Cucumber fruit is usually green and warted, however, white and yellow cultivars and smooth skinned green cultivars are available (Bassett, 1986).

b.The economic importance and uses of cucumber.

Cucumber is a major vegetable crop and is grown extensively in almost all parts of the world including Iran and the UK. In 1986, commercial plantings of cultivated cucurbits occupied 1.39 million ha in total of more than 70 countries; of these 39.7% were pumpkins, squash, and gourds, and 69.3% cucumber and gherkins (Table.1b).

The fruits of the cucumber plant are eaten as a salad vegetable before they are fully mature and are usually peeled. In the East they are eaten as a cooked vegetable. The young fruits, usually of small fruited cultivars are pickled as gherkins, the smallest for mixed pickles and small to medium sized fruits for dill pickles (Purseglove, 1968 ; George, 1985).

The seed kernel are occasionally eaten and yield an edible oil. The young leaves are eaten as salad or cooked as spinach in Indonesia and Malaya (George, 1985).

One of the uses of cucumber, not reported in the literature, is for reducing the effect of salty water getting into the eyes during swimming in the mineral lakes for therapeutic purposes in Orumiyeh, Iran. Due to the high concentration of salt in water and entire body being almost

plastered with salt, a cucumber is broken and the freshly exposed part is gently rubbed over the eyes. This is done as many times as necessary as the cucumber is available cheaply.

Table 1.b: Distribution of cucurbits in harvested areas and production in different parts of the world. Data from FAO yearbook 1986. Figures for the UK in brackets.

World regions	Harvested area		Total Production	
	1000 ha	% of total	1000 mt	%of total
Cucumber & Gherkins				
All developing countries	469	56	6332	51.5
Asia	442	52.7	6707	54.5
All developed countries	369	44	5971	48.5
Europe (UK)	122(81)	14.6(9.7)	259 (81)	21.1(0.7)
North Central America	75	8.9	971	8
Africa	24	2.9	386	3.1
South America	3	0.4	47	0.4
World total	838	100	12304	100
Pumpkin. Squash. Gourd				
All developing countries	380	68.8	4348	69.1
Asia	221	40	2787	44.3
All developed countries	173	31.3	1941	30.9
Europe	130	22.6	1318	21
South America	76	13.8	765	12.2
Africa	71	12.9	978	15.6
Central America	3	7.8	282	4.5
World total	552	100	6288	100

c. Cucumber production in the UK and in Iran.

Cucumber is warm-season crop and is intolerant of frost. However, because of their short growing season they are grown almost everywhere in the world. Ideal growing temperatures are near 30°C (86°F)(Hartmann et al, 1988).

Cucumbers were once a very seasonal crop and a single crop per year was common in most locations, but now several crops are grown in a year in many areas. In warm countries cucumbers are brought into cropping without the aid of artificial heating.

In UK, however the case is different, and in order to secure a good supply of cucumbers, even during the warmest seasons of the year, artificial heat and protection is indispensable (Andrieux, 1977).

In Iran, it is grown in the field and it can also be brought into cropping during the autumn and even in winter in the warmer regions of of the country.

Cucumbers are propagated from seed and planted when soils are warm and frosts are not a threat. In some areas, plastic tunnels and hot caps are used for very early production to secure better prices.

d.Cultivars of cucumbers.

Numerous cultivars of cucumber have been developed in many parts of world differing in size and shape of fruits; thickness, spininess and colour of rind, ranging from whitish-green to dark-green, others turning yellow or rusty when mature (Lower & Edwards, 1986).

The cucumber cultivars can be classified in different ways:

a. According to the fruit appearance and production methods.

(Purseglove, 1968; George, 1985):

1. Field cucumbers with white or black spines. These cucumbers are distinct from other types, because of their relatively thin, smooth skin and uniform green colour. The unique flavour tends to be milder and sweeter than other types (Asgrow Seed Company, 1984).

2. English or greenhouse cucumbers. There are three fruit types for this purpose. Dutch types are grown extensively in Europe and Canada and to a lesser extent in the U.S. They are characterized by ratio (L/D) in excess of 6.0. Dutch hybrid cultivars are parthenocarpic with gynoecious expression and high yield potential. Japanese greenhouse cucumber have L/D ratios of about 5.0 but differ from the Dutch type in that most hybrids are monoecious. This type in addition to greenhouse production also is grown in the open field on trellis (Asgrow Seed Company, 1984).

3. Sikkim cucumber of India with orange brown to reddish-brown fruits (Everett, 1981).

4. Pickling cucumbers with small fruits used for production of gherkins.

b. According to the ways the fruit are used.

1. Processing (pickling) cultivars.

Pickling cucumbers are processed from both fresh and brined cucumbers. Fruits are cylindrical in shape with blunt end angular, and warty. A length to diameter (L/D) ratio is about 3.0 and with a light to medium green colour.

2. Slicing types (Fresh Market).

Slicing type cucumbers are used almost exclusively as fresh cucumbers. Slicing cucumbers are always white spined and are characterized by smooth, symmetrical fruit with glossy, dark green skin. The fruits tend to be long and tapered, with widely scattered, less pronounced warts and slow seed development.

3. Greenhouse cucumbers (see above).

Hybrid cultivars providing greater yields through more abandant fruit set and gynoecious characteristics, and disease resistant are becoming increasingly important. The requirements for the fresh market and for processed cucumber are also quite different. Earliness and disease resistance are important for either purpose, but preferences differ for fruit shape, surface skin features, and spine colour. Cucumbers for processing should have lighter green colour and thin skins, should be blunt and short with a small fruit length to cross-sectional diameter ratio , approximately 2.5 to 3.0, compared to 4.0 or more for the long tapered, thick-skinned, darker green fresh market fruit (Hartmann et al, 1988).

Cucumbers for pickling also are characterized by their warty surface, Presently most cultivars have white spines, although black spine varieties were formerly preferred, because they provided an attractive final product (Hartmann et al, 1988).

2. In vitro techniques in crop production, plant physiology and plant breeding.

The vegetative propagation of plants has been practised for centuries and although many improvements in conventional methods have been introduced over the years, the more recent application of tissue culture techniques (i.e micropropagation) has considerably expanded both their scope and potential (Hussey, 1983).

In vitro propagation, generally referred to as microprpagation is a specialized method of propagation in which very small pieces of plant tissues are regenerated in an artificial medium under sterile conditions. The aim of all plant propagation is to multiply the numbers of a desirable phenotype/genotype; in micropropagation, this objective can best be achieved by two pathways:

I. By using unexpanded nodes (shoot tips) or expanded nodes with axillary buds.

II. By induction adventitious regeneration.

The distinction between the two main pathways of vegetative reproduction is important with regard firstly to the genetic stability of the propagules during multiplication and secondly, to their susceptibility to induced mutation or transformation in any form of genetic manipulation (Yeoman , 1986).

a. Axillary bud propagation (Micropropagation).

All plants grow through the action of their apical and secondary meristems. The apical meristem is an actively dividing dome of tissue located on the extreme tip of the shoot and usually measures 0.1 mm in diameter and about 0.25-0.30 mm in length. The totipotency exhibited by this apical meristem and adjacent shoot tip region has formed the basis of commercial clonal propagation for numerous plant species. Cultivated meristems may regenerate plants through axillary branching. Plants developed using this technique retain the genetic composition of the mother plant (Dixon, 1985).

The production of plants from axillary buds has proved to be the most generally applicable and reliable method of in vitro propagation (George & Sherrington, 1984).

Two methods are in use:

- Shoot tip culture.

- Single node culture (in vitro layering).

With shoot tip culture a shoot tip isolated, from which the axillary buds in the axils of the leaves develop, under the influence of a relatively high cytokinin concentration. This high cytokinin concentration suppresses the apical dominance and allows axillary buds to develop. If a shoot tip has formed a number of axillary or side-shoots, these can then transferred onto fresh media containing cytokinin; new shoots form . These shoots regenerate roots adventiously either in the same media or in fresh ones. and the resulting plantlets planted out into the soil (Pierik, 1987).

Single node culture is another in vitro technique that can be used for propagation of plants from axillary buds. This method is similar to some conventional propagation methods such as layering in vivo. Each axillary bud can be isolated and placed on the nutrient medium; this bud then induced to develop in vitro (Pierik, 1987).

b. Adventitious regeneration.

Under certain conditions the somatic tissues of higher plants are capable of regenerating adventitious shoots via (organogenesis) or embryos (embryogenesis)(Hussey, 1986).

Organogenesis may occur directly from explants, or indirectly from callus produced on the explant.

1. Organogenesis (direct and indirect).

In direct organogenesis the adventitious shoots arises directly from the tissues of the explant and do not develop within or on previously-formed callus. However direct shoot formation may be accompanied by some proliferation of unorganised tissue known as callus. Tissue of this kind should only be used as explant material if the generation of variation is the objective. Callus can usually be reduced by adjustment of the concentration of the growth regulators used. A closely packed mass of shoot primordia may be mistaken for organised callus (George & Sherrington, 1984).

The induction of direct shoot regeneration depends on the plant organ from which the explant was derived and above all , on the plant species. In some genotype, adventitious shoots arise in vitro on pieces of tissue derived from various organs (e.g leaves, stems, flower

petals or roots), while in others they arise only on a limited range of tissues such as bulb scales, seed embryos or seedling tissues (George & Sherrington, 1984).

Levels of growth-regulating substances, particularly auxin, higher than those necessary to stimulate the direct formation of adventitious shoots generally fail to produce shoots, or may only induce roots from which plants cannot be regenerated (Malepszy et al, 1983; George & Sherrington, 1984) . This may not mean that plants could not be regenerated from recalcitrant callus under any circumstances, but simply that a different medium, cultural environment or set of growth regulator treatments is necessary (George & Sherrington, 1984).

Adjustment of the growth regulators in the culture medium can bring about shoot formation in callus from a very large number of species. Inception of roots and shoots is most frequent in tissues that have been recently isolated, and morphogenetic capacity generally declines with time as the tissues are subcultured. However some callus cultures have maintained their regenerative ability over long periods (George & Sherrington, 1984). An organogenesis which is occurring under the above mentioned condition is called indirect organogenesis.

Normal callus cultures produce shoots relatively slowly, but from some plants, and certain explants, under conditions that are not yet understood, callus can be initiated which has an especially high ability to regenerate shoots or somatic embryos (George & Sherrington, 1984).

2.Embryogenesis (direct and indirect).

In the normal seedling cycle, embryogenesis proceeds from the single-celled zygote to a fully developed embryo. The ability of plants to produce embryos is not always restricted to the

development of the fertilized egg; embryos from cell, tissue and organ cultures may occur either directly or indirectly. The direct mode of embryogenesis involves the formation of an asexual embryo from a single cell or group of cells on a part of the explant tissue without an interving callus phase. Such occurrences are notable in Citrus, where pre-existing nucellar tissue cells give rise to nucellar embryos (in vivo and in vitro) and in various cases where cultured immature embryos exhibit budding. Also, cases have been reported such as in Ilex aquifolia and Rananculus sceleratus, in which the epidermal stem cells give rise to adventitious embryos (Dixon, 1985). somatic and haploid embryos can also be induced to form in cultured tissue (Bengochea & Dodds , 1986), this process is termed adventitious, asexual or somatic embryogenesis; which is in many respects analogous to zygotic embryogenesis.

In the literature somatic embryos are referred to by many names such as embryoids; embryo-like structures, adventitious or vegetative embryos. (Pierik, 1987).

The development of somatic embryos and plantlets from an explant can be summarized as follows (Pierik, 1987):

Differentiated cells should first be de-differentiated, after which they start to divide. In this way a non-organized mass of vacuolated parenchyma cells arises. This is then transformed into cytoplasm-rich cells which become embryogenic . The embryogenic cells from which embryoids are visually derived show a number of common features, which are characteristics of rapidly dividing cells. These include, small size, dense cytoplasmic contents, large nuclei with prominent enlarged nucleoli, small vacuoles and a profusion of starch grains. Their histochemistry and ultrastructure are suggestive of intense RNA synthesis and metabolic activity. The development of an embryo from the embryogenic cells is usually accomplished by the reduction of the concentration of auxin in the medium (Pierik, 1987).

The production of somatic embryos in the cell suspensions and callus tissue can take place either inside or on the periphery of the callus at the same time embryogenesis from nucellus tissue, hypocotyls of germinating plants, and from somatic embryos has also been described. Ammirato et al (1983) found the following to be good starting material for somatic embryogenesis: parts of flowers, zygotic embryos, anthers, pollen grains and endosperm tissue. On the basis of the fact that embryos were seen to originate from nodular or bud-like masses of tissue, it was concluded that somatic embryos were not unicellular in origin. Some authors mention that somatic embryogenesis in fact takes place from a single cell, which segments and so pro-embryo-like cell complex (Pierik, 1987).

Two different types of somatic embryogenesis can be distinguished (Evans et al,1981; Ammirato, 1983; Ammirato, 1986):

Direct:

In this case an embryo arises directly from a cell or tissue without previous callus formation. The cell from which the embryo develops are called pre-embryonic determined cells . The starting material for this type of embryogenesis is already completely rejuvenated. Examples are nucellus tissues of Citrus species, which have a tendency for polyembryony and epidermal cells of hypocotyls (wild carrot, Ranunculus, Sceleratus, Linum Usitatissium, Brassica, (Pierik, 1987).

Indirect:

This type of embryogenesis occurs in both callus and suspension cultures. Because they are not formed on tissues of original mother plant, shoots (or other organs) are said to be

regenerated indirectly when they are formed on previously unorganised callus or in cell cultures (George & Sherrington, 1984).

The cells from which the embryo arises are called embryogenically determined cells and form embryos when they are induced to do so (induced embryogenically determined cells). With this type of embryogenesis, differentiated cells must firstly be de-differentiated and then redetermined as embryonic cells after division. Auxins (and sometimes cytokinins) are of great importance in this process. With this method, complete rejuvenation must first take place. Examples giving rise to somatic embryos in this way are: secondary phloem of carrot, leaf explants of Coffea arabica, Petunia hybrida, and Asparagus officinalis (Pierik, 1987).

c. Some special considerations in vitro cultures.

I.Transitions between juvenile and the adult phase (e.g. precocious flowering in vitro).

One of the most dramatic examples of the alteration of form of a plant occurs at the time that the plant makes the transition from juvenility to maturity (phase change). For example, in many angiosperms, the plant produces root, stems, and leaves for a long period of time. Then, at some point in its life cycle, it ceases vigorous vegetative growth and begins a series of transformations leading ultimately to the production of the reproductive organ, the flower (Galston, 1964).

In plants the transition to flowering marks the beginning of reproductive development during which meristems produce flowers rather than leaves, stem and associated vegetative structures (Meeks-Wangner,et al,1989). Internal signals related to developmental age or external signals such as daylength or temperature can induce the onset of flowering, and there is evidence that both promotive and inhibitory substances are involved in the inductive process

(reviewed by Bernier, 1988). It is well established that some of these substances are generated in leaf tissues (Meeks-Wangner, et al, 1989). but other plant organs also have been observed to influence the initiation, and development of flowers (Bernier, 1988).

Poething (1988) points out that the transition from a juvenile to an adult phase of shoot growth in plants involves coordinated changes in a large number of morphological and physiological traits, including the ability of the plant to undergo sexual reproduction, enviromental, nutritional and chemical factors influence this process (phase change).

Although the above factors have been extensively studied for many years, the nature of the transition to flower has not yet well understood (Allsopp, 1965; Zimmerman et al, 1985; Poething , 1988).

II. Somaclonal variation in cultures.

If certain combinations of plant growth regulators is added to culture media, disorganized callus growth will occur (Lindsey & Yeoman, 1985). Plant cells can be maintained in this state for long periods, either on solid or suspended in liquid media, with routine subculturing. Alternatively, by altering the balance of growth regulators, it is possible in many cases to regenerate intact plants (Walker and Gingold, 1988). One consequence of passage of cells through even a brief callus phase is appearance of variability. This variability is known as "somaclonal variation" and can be manifested in many ways (Walker and Gingold, 1988).

Somaclonal variation has been widely described both in cultured cells and for plants regenerated from callus (Semal, 1986). The more obvious examples involve changes in chromosome number and structure of regenerated plants, resulting in morphologically

abnormal plants, and these are generally of no useful value (Walker and Gingold, 1988). It is also possible to obtain variants with apparently normal chromosome complements, which shows useful differences in agronomic characters. Successful selection can be achieved for specific traits such as resistance to herbicides (Chaleff & Parsons, 1978; Jones, 1985) and salt (Nabors et al, 1975).

d. Germplasm storage.

Germplasm can be conserved efficiently by seed storage . However for recalcitrant seeds and the plants which do not produce seeds, numerous methods have been devised and tried out to achieve short and long-term preservation of germplasm.

Tissue culture methods now provide a valid alternative to more conventional methods for storage of germplasm where it is necessary to conserve vegetative material (Henshaw, 1982).

Two classes of methods in respect of the aim and period of storage have been considered. The first is aimed at extending the interval between subcultures, therefore it is called low-growth storage or other synonymous terms, and the second is to maintain the plant in non-growing state at very low temperatures (cryopreservation).

I. Slow-growing cultures.

The term "slow growth" is used to cover "growth limitation" or "minimal growth" and other terms which are brought about by a modification of the culture conditions (Withers, 1986).

The aim with slow-growing procedures is to extend the subculture interval from one month to as long as possible (Henshaw, 1982; Aitken-Christie & Singh, 1987).

Slow-growing cultures can be particularly useful when space is limiting in the culture room, when labour is in short supply for transfer or during holiday periods (Aitken-Christie and Singh, 1987).

There have been several approaches to limit the growth in the plant tissue cultures. Three principale methods are in use:

1) The physical conditions of culture can be altered, e.g., temperature or the gas composition in culture vessels. 2) The basal medium can be altered, for example, using sub- or supraopimal concentrations of nutrients. 3) The medium can be supplemented with growth retardants (e.g., abscisic acid) or osmo-regulatory compounds such as mannitol and sorbitol (Henshaw, 1982; Withers, 1983; Dodds and Roberts, 1985).

II. Non-growing cultures.

Germplasms can also be stored in cultures in a non-growing state (cryopreservation).

Cryopreservation (Greek, kryo= frost) is literally "preservation in the frozen state "

In essence this method involves rapid cooling of biological material to very low temperatures, and storage under conditions which will preserve the ability to resume growth when brought back to normal temperatures. Most biological material will not endure such conditions unless previously treated with cryoprotectants, compounds which protect it during deep freezing (Mayer & Mayer, 1989).

Actively growing plant tissues appear to be more resistant to freezing than quiescent ones, with some exceptions such as seeds or dormant buds. Therefore in most cases a "pre-growth" period is allowed before freezing. This may be considered as the first step in a cryopreservation protocol (Mayer & Mayer, 1989).

During the next step, cryoprotection, the tissue is exposed to a complex mixture of highly hypertonic solutions. Among the most frequently used compounds are dimethylsulphoxide (DMSO), or glycerol mixed with sugars, sugar alcohols or high molecular weight polymers such as polyethyleneglycol 6000. Different ratios of the compounds and different lengths of exposure are used for different tissues, the treatment being rather empirical (Henshaw, 1982; Bhojwani and Razdan, 1983; Mayer & Mayer, 1989).

The third step is the actual cooling, usually in liquid nitrogen. Optimal rates of cooling must be determined empirically for each tissue (Kartha, 1985; Withers, 1986). Subsequent storage is at very low temperatures, below -100°C (Mayer & Mayer, 1989).

A further critical stage in cryopreservation is the return of tissue to normal temperatures. Warming should be such as to avoid recrystallization of intracellular ice. Generally rapid warming at 40°C is recommended. The thawed material is transferred to the recovery stage for resumption of growth and development. Each tissue appears to respond differently to the cryoprotectants and the rates of cooling and rewarming (James. , 1983; Mayer & Mayer, 1989).

e. Organ culture in studies of sex expression in monoecious genotypes.

Sex expression in plants has been the subject of many investigations, which have been studied quite extensively in vivo in the past, and a considerable amount of information both on the genetic basis of sex determination (reviewed by Westergaard, 1958) and on its modification by environmental conditions and by chemical agents, mainly plant growth regulators, has accumulated (reviewed by Heslop-Harrison, 1957; Lang, 1961).

The technique of in vitro culture of young floral buds offers several advantages in investigating the various mechanisms involved in floral morphogenesis (Rastogi & Sawhney, 1988):

1) The direct role of nutrients and growth regulators in floral development can be determined by eliminating the interference of substances translocated from other plant parts.

2) A variety of surgical experiments can be performed to elucidate the determinative events, the regulative behaviour, and regenerative potential of entire buds and individual organs.

3) The regulative influence of one organ type on the initiation and development of another can also be determined by surgical manipulation in vitro.

4) The nature and timing of floral organ determination, and the control of cytodifferentiation in each organ type can be investigated by culturing individual organ primordia.

5) The autonomy of floral buds, i.e whether the pattern of organ initiation and development is independent of the rest of the plant, can also be tested.

Three types of primordia have mainly been investigated (Rastogi and Sawhney, 1988):

a) young inflorescences b) individual flower buds and c)floral parts

f. Other in vitro techniques.

There are other in vitro techniques which were explained above, and these are used for different purposes. Details of these techniques are unnecessary, but some simple generalizations may be helpful.

I. Protoplast cultures.

One of the most significant developments in the field of plant tissue culture during the last decade has been the isolation, culture and fusion of protoplasts (Cocking, 1972).

The techniques are especially important because of their implications in studies of plant improvement by cell modification and somatic hybridization (Bajaj, 1974). Isolated protoplasts also offers a means of tackling various fundamental and pragmatic research problems in experimental plant biology (Bajaj, 1977).

The availability of protoplasts makes it possible, for example, to study plasma members and absorbtion without interference of the cell wall (Gamborg, 1973). The biosynthesis and deposition of the cell wall can be investigated in a more elegant manner than has previously been possible and removal of cell wall may greatly faciliate extraction and isolation of organells and macromolecules.

Although protoplasts may be very important in elucidation of details of cell structure and function, their greatest importance promises to be their use in somatic cell hybridization and possibly host-parasite investigations (Gamborg, 1973).

II. Anther and pollen cultures.

Plant breeding by doubled-haploid in vitro cultures has shown its value. It not only shortens the time needed to create a new variety, it also can elicit new information on the quality of the selection process at the pure line level. It offers the possibility of measuring the improvement made in a new genotype at the homozygous stage (Nitsch, 1983).

Anther and pollen culture is important as a mean to study physiological and biochemical processes in asexual embryogenesis. It also is a useful technique in the production of haploid plants for basic and applied research in breeding, mutation and genetic development in plant tissue culture techniques offer possibilities of introducing into plants variability that could be utilized for crop improvement. Haploids with their unique genomic constitution, have potential for accelerating the production of homozygous new varieties (Harda. & Imamura , 1983; Zapata et al, 1983).

III. Embryo rescue.

One of the key problems in present-day horticultural and breeding practices is that wide crosses have not yielded any agricultural beneficial hybrids. This is usually attributed to the existence of crossability barriers which apparently interfere with the transfer of useful alien

(foreign) gene between unrelated species and genera (Evans et al, 1986; Raghavan, 1986). Crossability barriers encountered in hybridization practices may be due to sexual incompatibility or to hybrid break down (Raghavan,1986).

Different observation have shown that embryos of wide crosses invariably suffer from a lack of continuing supply of essential nutrients to complete their development in the ovule (Raghavan. , 1986).

The purpose of this section is not to consider the cause for hybrid inviability in wide crosses, therefore we will follow the subject with methods to rescue embryos from unsuccessful crosses.

The basic premise upon which embryo rescue options have been attempted in wide crosses is that the integrity of hybrid genome is retained in the stalled embryo and that is potential to rescue normal growth may be realized if it is supplied with nutrient substances, that are known to promote growth of embryos. This assumption has been born out by excision and culture, under aseptic conditions, of hybrid embryos in an artificial nutrient medium (Raghavan, 1986).

There are different ways which are used for hybrid embryo rescue (Raghavan, 1986):

-In some instances, the hybrid embryos are implanted on the normal endosperm, which are then essentially initiate a nurse culture.

-In a limited number of cases, efforts directed toward rescuing hybrid embryos by culturing ovules and ovaries seem very promising.

-A potentially useful, but largely unexplored method is to obtain transplantable seedlings from unsuccessful crosses by organogenesis or by somatic embryogenesis.

IV. Production of secondary metabolites.

A large number of commercial valuable chemicals in use today are delivered directly from plant material. The types of compounds, thus obtained fall into five broad categories of applications: drugs, flavours, perfumes, pigments, and agriculturally useful chemicals. Biochemically , the great majority of compounds are secondary metabolites (Walker & Gingold, 1988).

According to a report of the U.S. congress (1983) pharmaceutical derived from plants and intermediates represents an annual U.S market of U.S $ 9 billion at consumer level, while aroma components represent a worldwide market of U.S $ 1.5 billion (Sahai & Knuth, 1985).

25% of all prescription pharmaceutical used in the UK, in 1988 were derived directly from plant material (Walker & Gingold, 1988).

Although plant tissue culture techniques have been in existence since the early 40·s significant advances in culture technology took place only over the last decade, when Mitsui Petrochemical announced of japan industrial process in 1982 in the production of Shikonin. This red dye was the first chemical product to be made via plant tissue culture. The compound was used as anti-inflammatory pharmaceutical and as ingredient for cosmetics (Curtin, 1983; Sahai & Knuth, 1985; Fowler, 1987; Walker & Gingold, 1988).

This development has done much to boost the technology (application of tissue culture for production of secondary metabolites).

Plant tissue culture is now sufficiently well established as a third manufacturing approach, although it may be rejected for a particular product on commercial considerations (Fowler, 1987).

In order for cell cultures to be used as commercial sources of secondary compounds, the in vitro production must be comparable to or exceed the amount produced by the intact plant. Several reports have been published indicating yields approaching or exceeding yield from the whole plant (Dodds & Roberts, 1985).

As Yeoman (1987) has pointed out sometimes cultured products are more efficient than the intact plant. For example, cultures of Coleus blumei accumulate nine times more Rosmarinic acid than the plant.

In spite of the technical difficulties in producing high value secondary metabolites in vitro, we are in agreement with Robins & Rhodes (1988) that the technology is of interest even though it may not be applicable immediately.

V. Meristem culture for virus elimination.

Internal infections caused by viruses, mycoplasmas, bacteria and fungi can be very difficult to combat. It is virtually impossible to eliminate the plant of these internal pathogens by the use of chemicals. It is sometimes possible to suppress the virus multiplication by the use of relatively expensive compounds in the nutrient medium (Walkey, 1980; Kartha, 1986; Pierik, 1987). The addition of antibiotics to counter internal bacterial infections is equally ineffective.

In contrasts to what thought previously, viruses can also be transferred during generative propagation; about 600 viruses are known of which at least 80 can be transferred in the seed (Pierik, 1987), viruses, bacteria and fungi are nearly always transferred by vegetative propagation.

There is available concentration of virus in the cells of an intact plant. In particular , apical shoot and root meristems are liable to contain few viruses or may even be virus-free (George & Sherrington, 1984).

Some care must be exercised in the terminology we use . The term "virus-free " means that a given plant is no longer infected with any virus that can be detected by one or more virus-indexing techniques: Unknown viruses, however, may still be present within the plant tissues. It is preferable to apply the term "virus-tested " to these plants, since they may not literally be virus-free (Quak, 1977; Dodds & Roberts, 1985).

In vitro techniques (meristem culture) rely on aseptic procedures for the elimination and exclusion of contaminating organisms which would otherwise damage the plant tissues, can be quite adequate for many purposes. But it can be only regarded as a first embankment (line) of defence where plant health is a major consideration, since certain contaminants, especially systemic , bacterial, mycoplasmas and viruses, can be undetected unless special indexing procedures are adopted (Henshaw, 1984).

There are five available methods of producing virus-free plants (Pierik, 1987):

1. Heat treatment; 2. Meristem culture; 3. Heat treatment followed by meristem culture; 4. Adventitious shoot formation followed by meristem culture; 5. Grafting of meristem (micro-grafting) on virus-free seedling.

It is now apparent that the present in vitro techniques do not , alone , quarantee the elimination of systemic pathogens, therefore the existing methods should be improved. The improvements can be achieved by using the therapy treatments or in vitro techniques combination with appropriate indexing procedures, which will be expensive and time-consuming. It is essential to eliminate pathogens from a relatively high proportion of plants if the overall process is to be an economic proposition (Henshaw , 1984).

3.Current state of in vitro work in cucumber.

I.Axillary bud propagation.

The first paper to the in vitro work with cucumber was published was about ten years ago (1979) by Handley and Chambliss. They succeeded in raising complete plants from axillary buds of the plant. A medium containing 0.1 mg NAA and 0.1 mg Kinetin, gave the best results. The best balance between root and shoot growth occurred on the same media.

In 1982 Fortunato & Moncini in an abstract claimed that culturing shoot tips and lateral apices of cucumber on media containing 1.0 ppm IAA, 1.0 ppm 2ip plus 0.025 ppm GA3 gave 10 shoots from each explant in 12 days.

Since then no work on either axillary buds or shoot tips has been reported, therefore reports on meristem culture buds for purposes other than propagation have been published.

Giesmann & Sabharwal(1969) have studied the flowering potential of cucumber plants obtained from apical meristems of Long Green variety under in vitro conditions.

Rute et al (1978) studied the influence of physical and chemical cultivation factors on growth and morphogenesis of apical meristems of cucumber plants under in vitro. Their experiments show that cultivation of meristems in a liquid nutrient medium on filter paper bridges significantly accelerates the development of regenerants. Amongst the media tested MS hormone free liquid medium gave the highest plant regeneration frequency (75-85%) from cucumber apical meristems and ensures of their normal development. In their experiment the photoperiod did not affect the regeneration of plants from cucumber apical meristems.

II. Adventitious regeneration.

- Direct organogenesis

Direct regeneration of adventitious bud occurred, (Bergervoet and Custers, 1986) from big (i.e. 1-3 cm) explants of cucumber hypocotyl sections. These explants were incubated on MS hormone free medium with 3.5% sucrose, 0.05% trypton, 0.8% Difco Bacto agar, 50 µM K and 0.5 µM IAA in 24 h light.

-Indirect organogenesis and embryogenesis.

A considerable amount of progress has been reported in the area of indirect organogenesis and embryogenesis of cucumber. Adventitious shoot formation from cotyledon tissue has been reported (Wehner & Locy, 1981; Novak & Dolezelova, 1982; Kim et al, 1988). Wehner and Locy, examined cultures of cotyledon and hypocotyl tissues from eighty-five cucumber genotypes and concluded that morphogenetic potential was dependent on genotype.

Kim et al (1988) did a further examinations on regeneration from callus. They studied the growth and differentiation of callus tissues derived from cotyledons of ten cultivars of cucumber.

Cotyledonary explants from all ten cultivars formed callus tissue on MS hormone free medium supplemented with 0.5 μM 2,4-D and 5 μM BA. Shoot development was achieved in three cultivars, on MS medium supplemented with 0.5 μM NAA and 5 μM BA. Reducing the BA concentration to 0.01 μM resulted in root formation on callus and on shoots transferred to this medium.

All cultivars gave the same response in tests of root formation. But shoot regeneration from callus culture of cucumber cotyledons was dependent on genotype.

Recently , plants have been regenerated from anther cultures (Lazarete & Sasser, 1982), leaf callus (Malepszy & Nadolska-Orczyk, 1983; Nadolska-Orczyk & Malepszy, 1984; Chee & Tricoli, 1988). hypocotyl segments (Sato et al, 1979; Wehner & Locy, 1981; Rajasekaran et al , 1983; Bergervoet & Custers, 1986; Ziv & Gadasi, 1986).

Only a few experiments have been reported on the isolation and culture of cucumber protoplasts. Coutts and Wood (1975) and Coutts (1977) isolated cotyledon and mesophyll protoplasts from two different cultivars in which sustained division, callus formation and root differentiation were obtained from mesophyll protoplasts isolated from the first true leaf. They reported less success for cotyledon-derived protoplasts. Lu and Cocking (1984) reported that while shoot formation was obtained from cotyledon protoplast culture of cucumber, no plant regeneration occurred. Plant regeneration from protoplasts of cucumber seems to be rather difficult. Fewer papers have so far been published on this topics, Orczyk & Malepszy, 1985 used leaf tissues; Jia et al, 1986 and Trulson & Shahin, 1986 used cotyledon as a source of

protoplasts. In each of these studies the number of regenerated plants was very low and most of the regenerated plants are abnormal.

In a recent paper Colijn-Hooymasd et al, 1988 investigated upon the differences between leaves and cotyledons as sources for protoplast isolation and plant regeneration.

In all their experiments, protoplasts only have occasionally divided in liquid medium (PE 2%), whereas in agarose bead and disc culture high plating efficiencies were obtained (33 to 42% for protoplasts from leaves and 39 to 42% of protoplasts from cotyledons).

Embryogenesis was observed more frequently yellow calli than white ones. Embryo development was obtained both from cotyledon and leaf protoplasts. However, only in the case of leaf protoplasts were morphologically normal plants obtained. Chromosomes studies showed that the ploidy level was higher in cotyledons than in leaves. With the procedures they established, shoots were obtained three months after protoplast isolation .

In spite of these advances the authors (Colijn-Hooymasd et al, 1988) were not satisfied with the regeneration frequency obtained.

It can be concluded from the literature that the genotype and source of explant are more likely to be critical for micropropagation and plant regeneration in vitro than the exact composition of the medium.

III. Culture of immature buds.

Although attempts to culture floral explants were first made as early as 1942 by La Rue, the first report of successful flower bud culture was that of Galun et al (1962, 1963). Floral buds of male , female and bisexual lines of Cucumis sativus were cultured to examine the role of growth substances in the differentiation of sex organs. Buds with well-developed sepal, petal and stamen primordia, and carpels as small depressions, were cultured on modified White's basal medium, containing coconut milk (CM), casein hydrolysate and sucrose, and supplemented either with indole-3-acetic acid (IAA) or gibberellic acid (GA3), or both. This medium supported the growth of all three types of floral buds, although the growth was inferior to that of in vivo. In male buds, IAA not only promoted ovary development but also suppressed the growth of stamen primordia. GA3 counteracted the effect of IAA but had no effect on its own on the growth of sex organs. It was also noted that very young buds (0.3-0.5mm) that were potentially male tended to develop ovaries when cultured in vitro even in the absence of IAA, and the younger buds exhibited greater tendency for such change than the older (0.6-0.7 mm) ones. The morphogenesis of the potentially female and bisexual buds was, however, not appreciably affected by GA3 or IAA, either alone or in combination.

IV Embryo rescue.

Interspecific hybridization is used to transfer disease resistance carried by wild Cucumis spp of the African continent into the cultivated cucumber C. sativus L., which originates from the Asian continent. Custers and Kruit (1982) tried to apply their experience gained from their work in embryo culture to rescue abortive hybrid embryos.

They showed that their experience with vital embryos were not suitable to rescue hybrid embryos with breakdown during the proembryo and globular-shaped-stage.

Their success were more with embryos showing starvation or breakdown during the cotyledonary-stage, and with embryos which reached maturity, but they failed to germinate in soil. In both cases embryos were recovered and developed into plants by artificial culture. Such embryos, however passed the period of continued embryonic development in vitro with much more difficulty than the non-hybrid embryos. The same was found in some crosses, which yielded normally germinating seeds. They add that embryos without cotyledons do not endure excision of cotyledons, since they die immediately afterwards. They consider the culturing of the cotyledonary hybrid embryos with late-heart-shaped-stage as a best solution to the above problems.

Their attempts to develop the initiation of callus from various tissues of cucumber plants were unsuitable for proembryos. In all their embryo culture they have not obtained viable hybrid plant from a cross between a specious of the African group and C.sativus. Although great numbers of embryos were formed in such combinations, but they ceased growth at the proembryo or globular-shaped-stage.

4. Aim of this project.

1. To develop a reliable protocol for the clonal propagation of cucumber. Both as an enabling technique in F1 seed production and under certain conditions as a substitute for it.

2. To develop suitable procedures for the low labour-input maintenace of vegetative shoot or node cultures.

3. To study the effect of hormones on the sex expression in immature inflorescences in vitro.

4. To study the stability of a well characterized single gene controlled dominant trait; bitterness in plants regenerated from callus induced from heterozygous donor plants.

CHAPTER:TWO

MATERIALS AND METHODS

1.Plant materials.

Seeds of cv. Telegraph (improved) were obtained from Sutton's Seeds Ltd; seeds of Telegraph 314, Rebella (F1), Pepinex 69 (F1) and Marketer were obtained from Bookers Seeds Ltd; seeds of Telegraph (P1), Chipper (P2), Tele x Chipper (F1), Perfection (P1), Ottawa (P2) and Perfection x Ottawa (F1) were a gift of Dr J.W.M. Smith (Practical Plant Genetics).

The following vegetative parts of plants were used as explants a) cotyledons b) unexpanded leaves c) Shoot tips d) stem nodes with their axillary buds without their leaf f) immature inflorescences.

2. Chemicals and culture vessels.

Chemicals were of Analar grade where possible. Unless otherwise indicated explants were cultured on MS (Murashige and Skoog, 1962) hormone free media (basal media). Details of the composition of basal media are shown in Appendix 1. Basal medium was supplemented with various combinations of growth regulators, including auxins (2,4-D, IAA and NAA), cytokinins (BAP and kinetin) and 2-Chloroethylphosphonic acid (ethrel).

For experiments on organogenesis sterile plastic Petri dishes were supplied by Sterilin Ltd. Petri dishes were sealed with ethanol sterilised "Parafilm M"

Glass jars with plastic screw tops were supplied by Richardsons of Leicester. General purpose glassware was cleaned by firstly washing in hot water with detergent, then rinsing in tap water followed by distilled water before drying in an oven.

3.Preparation of nutrient media.

The media usually based on those of Murashige and Skoog (1962) hormone free media. The MS medium was obtained in powder form (pre-packed) from (Flow Laboratories Ltd, Irvine, Scotland) and was stored in a fridge . The medium was made up with distilled water.

All stock solutions were stored in 250 ml glass reagent bottles in fridges in the dark. Fresh stock solutions were prepared every two months.

Stock solutions of the plant growth regulator benzyladenine (BA) and Naphthyl acetic acid (NAA) were dissolved in 1-2 ml of 70% of ethanol.

Heat-stable plant growth regulators as well as other compounds were added before autoclaving, while heat labile compounds such as IAA and ethrel were filter-sterilised through a membrane filter of pore size 0.2 μm (Acrodisc, UK.) and added after autoclaving to the media after cooling.

To prepare 1 lit of liquid medium the appropriate volume of each stock solution (including growth regulators) was pipetted into a 1 lit conical flask on a magnetic stirrer, and distilled water added to about 900 ml. Sucrose was added to the distilled water to give a concentration of 30 g/l (before autoclaving). The pH was adjusted to 5.7 by using 0.1 M

sodium hydroxide (NaOH) or 0.1 M hydrochloric acid. Agar (Lab M code MC2) at 0.7 0r 0.8 % was added after pH adjustment.

The media were dispensed into a 1 lit volumetric flask and made up to 1 lit with distilled water. The agar was dissolved and mixed by heating. Samples of media (25-30 ml) were then dispensed into 175 ml glass jars with screw tops, then capped , labelled and autoclaved for 15 minutes, at 15 lbs/sq at 121°C. The jars containing the media were stored at room temperature and used within one week of preparation.

4. Explant preparation and surface sterilisation.

Seeds of the cucumber cultivars were submerged in 70% ethanol for 1 min and surface sterilised with 4% sodium hypochlorite (10-14% W/V available chlorine) plus 5 drops of Tween 80 for 5 mins.

To check for contamination the seeds were placed on 9 cm Petri dishes containing nutrient agar (Lab m, Nutrient Broth No2) and incubated at 25±1°C. For germination, the seeds were transferred aseptically to a half strength MS hormone free medium + 3% sucrose + 0.7% agar (Lab m code MC2) in 9 cm Petri dishes containing solid medium. The seeds were maintained in an incubator under a 16-h daily photoperiod at 25±1°C.

When seedlings were 13 days old, they were ready for taking shoot tip explants.

5. Methods of assessment; calculation of shoot doubling time.

Shoot doubling time (Flegmann and Wainwright, 1981) as calculated from the numbers of shoots at the beginning (N_0)and end (N_t) of several successive subcultures.

At each subculture the natural logarithm (ln) of the ratio $\frac{N_t}{N_0}$ was taken and added to the previous total. The sums of these logarithms $\ln \frac{N_t}{N_0}$ were then plotted against the time (t) that has elapsed from the start of the culture . If the resulting plot (ignoring an initial lag period) is a straight line then $N_t = N_0 e^{Kt}$ where K is the slope of the line.

Shoot doubling time (t_d) was obtained from the equation:

$$t_d = \frac{\ln 2}{k} = \frac{0.69}{k}$$

Shoot doubling time can therefore be calculated from the slopes of the straight lines. The slopes have been established either graphically or by regression analysis.

6. Establishment of plantlets in compost.

To establish the in vitro rooted plantlets to compost, they were removed from the proliferation medium, and washed free of agar in warm tap water and transferred to a potting medium combination of perlite and Fisons M2 compost (1:1).

To provide the necessary high relative humidity the plants were placed into the propagation boxes protected by plastic covers for the first ten days.

After that the plantlets were transplanted to No 9 plastic pots containing Fisons M2 compost. Finally plants were transferred to the glasshouse and grown a further 80 days.

7. Cytology.

Seeds of cucumber were set to germinate between moist rolled paper towels (Bowater-Scott, Bowatowel 22) at 25°C. Root tips after two days emerged. About 10 to 15 roots (5 to 15 mm long) were collected.

It is important to remind that root tips should be selected which are actually growing and contain a large number of division figures. We found that the more division figures could be found in root tips collected after 24 hours (collected at 4 p.m).

The excised root tips were placed in glass vials and pretreated in α-bromo naphthaline (2 drops in 10 ml distilled water) for 2 1/2 hrs. After rinsing 5 times with distilled water, fixation was taken place for 15 mins in Farmer's Fixative (3 parts absolute alcohol, 1 part acetic acid).

The fixative was simply poured off the material and then the root tips were hydrolysed with molar hydrochloric acid (HCl) at 60°C for 8 mins. The hydrolysed root tips then were transferred to Feulgen, aceto-carmine stain and stored for 2 1/2 hrs at room temperature in darkness (aliminium foil wrapped around the vial).

Individual roots were placed on a slide and only the blue distal portion, 1 to 2 millimeter in length were used for making the smear. A drop of lacto-propionic orcein (60%) was placed on the root and were squashed in the stain with a flat brass bar. A cover slip was placed on the slide and gently heated slide over flame of an alcohol burner. The heat of slide was tested by passing it against back of hand between passes through flame.

After placing several sheets of filter over slide, flattened preparation by pressing down on cover slip with thumbs. Thus the preparation became ready to be viewed under the light microscope.

The study of chromosomes pairing relations were made with metaphase, because chromosome configurations are distinct at this stage.

8.Histology.

Details of the procedures for fixing, embedding, sectioning and staining of small specimen (unexpanded leaves) are given in Appendix 1.

The samples first were fixed in FAA, formalin/ acetic acid/ alcohol(70% ethanol)(90:5:5) mixture for twenty hours and then dehydrated by passing through the Tertiary butyl alcohol (Fisons SLR grade, Loughborough, Leics, England) series (see Appendix 1). At 100% TBA, Erythrosin B was added to make the specimens more conspicuous before they were embedded in paraffin wax with Ceresin (m.p. 56°C, BDH chemicals Ltd).

Longitudinal Sections of the wax-embedded specimens of 6 um thick were cut to make ribbon with a Cambridge Rocking microtone (Cambridge Instrument Co Ltd., UK). Sections then floated on warm water to allow expansion, before fixation to glass slides with Haupt's adhesive (see Appendix 1) of a 3% v/v formation solution. Sections were left on hot plate to dry overnight, the slides then placed in glass baskets(10 per each) and after labelling the staining procedure was performed (see Appendix 1).

Slides were made permanent using one or two drops of D.P.S mountant contains 80% Histo clear on the specimen and covered with a big cover slip. For long term usage the slide must be bubble free. When the permanent slide were ready they then viewed under a Leitz orthomat (W. Germany and Olympus BH-2"Japan" light microscope)in order to follow the origin of the plantlets.

CHAPTER: THREE

Clonal propagation through shoot tip and node culture

Three major experiments were carried out with the aim of developing a reliable protocol for the clonal propagation of cucumber. Three cultivars were used throughout: Telegraph, Rebella and Pepinex. Telegraph is a commercially important cultivar in the UK; it is usually monoecious, but under some conditions it can be gynoecious. Rebella and Pepinex are both F1 hybrids; Both Rebella and Pepinex dominantly gynoecious, while Pepinex is predominantly gynoecious but under some conditions such as under low temperature it produces some male flowers.

The main object of the experiments was to optimise the hormone treatments, to investigate the importance of the type of explant, and to examine the effect of culture temperature on the proliferation rate on root induction and on callusing.

The experiments were set up as follows:

Experiment 1. The effect of hormone treatments.

a. Proliferation rate and shoot length.

b. Root induction and callusing.

Experiment 2. Choice of explants.

a. Three passages of 3 weeks each.

b. Three passages of 4 weeks each.

Experiment 3. Effect of temperature.

a. Choice of a suitable temperature for optimal shoot proliferation.

b. Choice of a suitable passage duration at 30° C.

RESULTS.

Experiment 1. Effect of hormone treatments on the proliferation rate and shoot length.

To investigate the effect of a cytokinin (BA) and an auxin (NAA) on shoot proliferation full factorial experiment was set up using 4 levels of each plant growth regulator (PGR)i.e. 0, 0.3, 0.4 and 0.6 μM alone and in combination. Shoot tips 3 to 5 mm long, were used as explants. The explants were taken from 13-day old in vitro grown seedlings under aseptic conditions and placed on the specified media in 175 ml glass jars with plastic screw tops and maintained in a growth room at 25±1°C with a day/night regime of 16/8 hrs with light intensity of 2.9 Wm^{-2}. There were 7 to 13 replicates; for each treatment, one to three explants were placed in each jar.

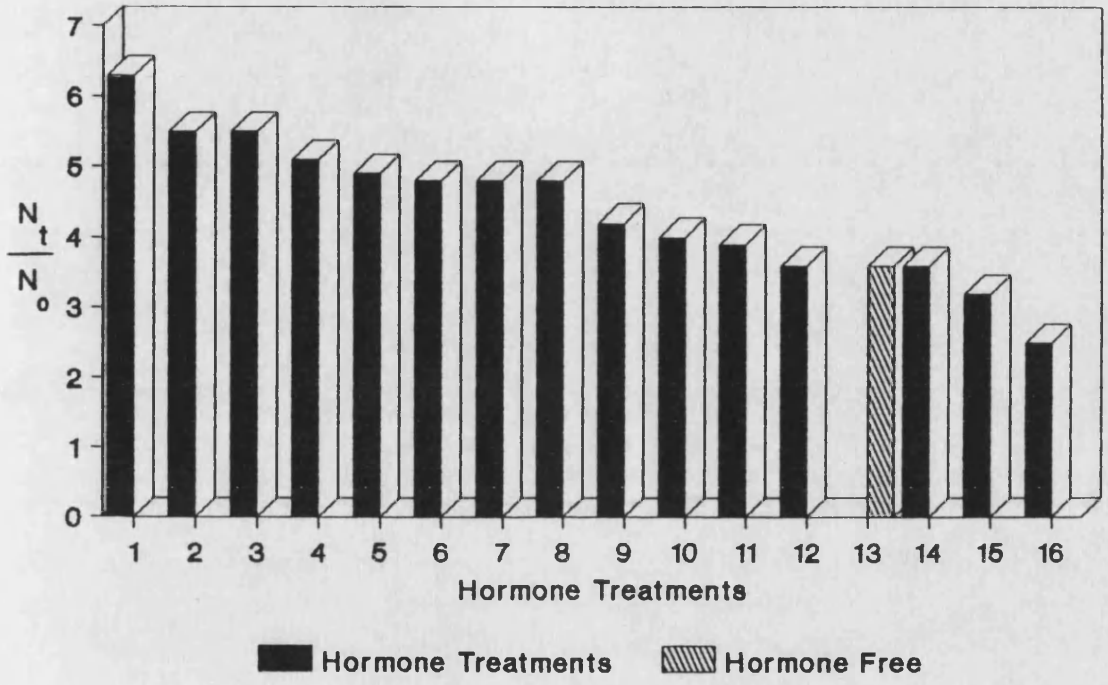
Evaluation was carried out after a single culture passage of 32 days in terms of the numbers of shoot tips and nodes produced. Cultures were also ranked according to shoot quality, taking into account the length of the internodes and the general appearance of the plantlets. Internodes shorter than 5 mm were considered being of low quality. The maximum length of the main shoot (from shoot tip to top of basal callus i.e. collar) was also measured.

a. The effect of hormone treatments on the propagule numbers and on shoot length.

The results are shown in Figs 1 to 9, and in Tables 2-4 (Appendix 2) respectively.

There were very significant differences between the proliferation rates on some of the treatments ($p=0.01$) as shown in Tables 2 to 4.

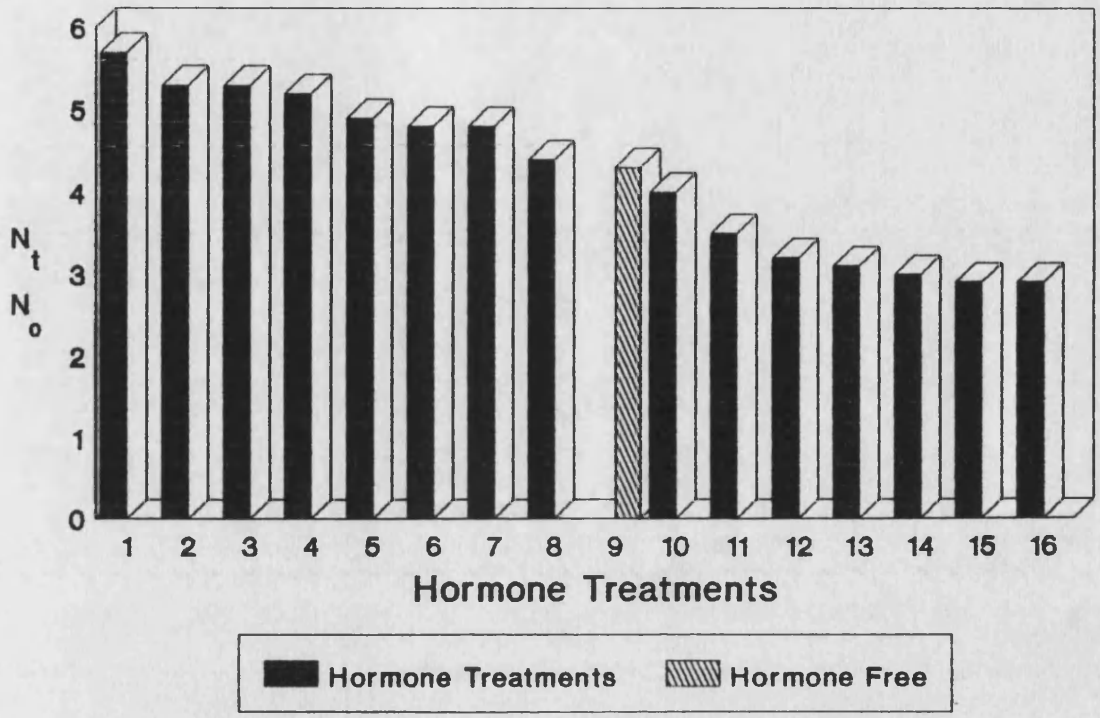
Fig.1. Effect of hormone treatments on propagule numbers in cv. Telegraph



Key
Treatments (μM):

1. 0.6 BA
2. 0.3 NAA
3. 0.3 NAA+ 0.6 BA
4. 0.4 BA
5. 0.4 NAA+ 0.3 BA
6. 0.3 NAA+ 0.4 BA
7. 0.3 BA
8. 0.6 NAA+0.4 BA
9. 0.3 NAA+ 0.3 BA
10. 0.4 NAA
11. 0.4 NAA+ 0.6 BA
12. 0.4 NAA+ 0.4 BA
13. Hormone-Free
14. 0.6 NAA+ 0.6 BA
15. 0.6 NAA+ 0.3 BA
16. 0.6 NAA

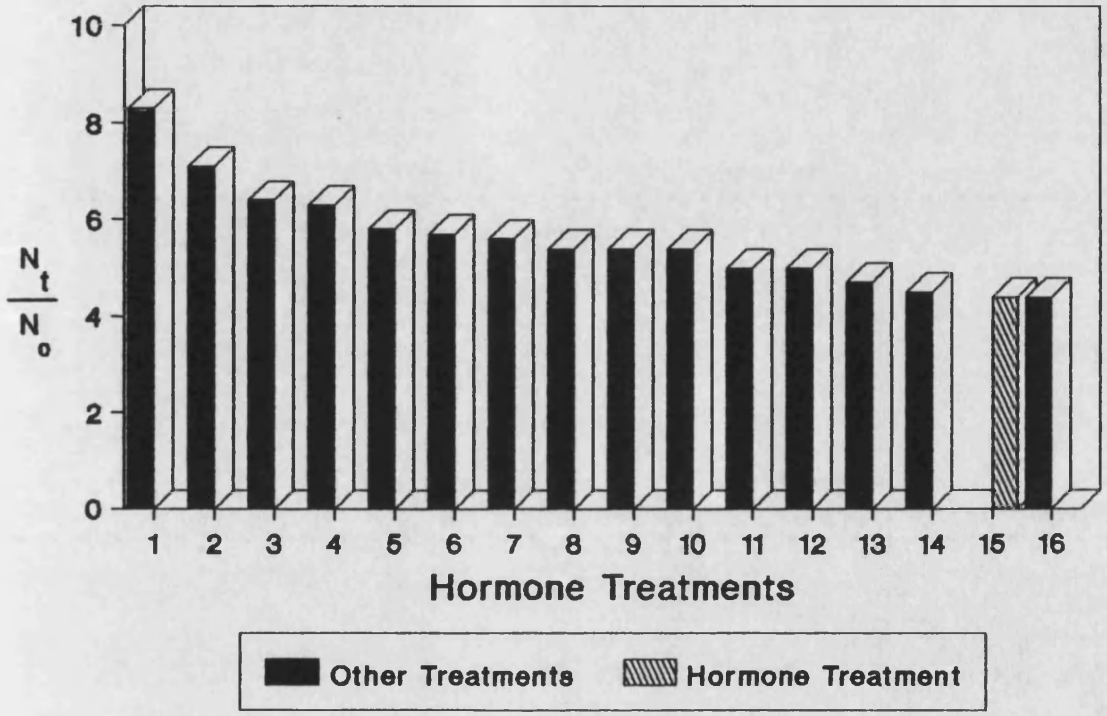
Fig 2: Effect of hormone treatments on propagule numbers in cv. Rebella



Key
Treatments(μM):

1. 0.6 BA
2. 0.3 NAA+ 0.6 BA
3. 0.3 NAA
4. 0.4 NAA+ 0.4 BA
5. 0.4 BA
6. 0.3 NAA+ 0.3 BA
7. 0.3 BA
8. 0.4 NAA+0.6 BA
9. Hormone-Free
10. 0.6 NAA+ 0.4 BA
11. 0.6 NAA+ 0.6 BA
12. 0.3 NAA+ 0.4 BA
13. 0.6 NAA
14. 0.4 NAA
15. 0.4 NAA+ 0.3 BA
16. 0.6 NAA+ 0.3 BA

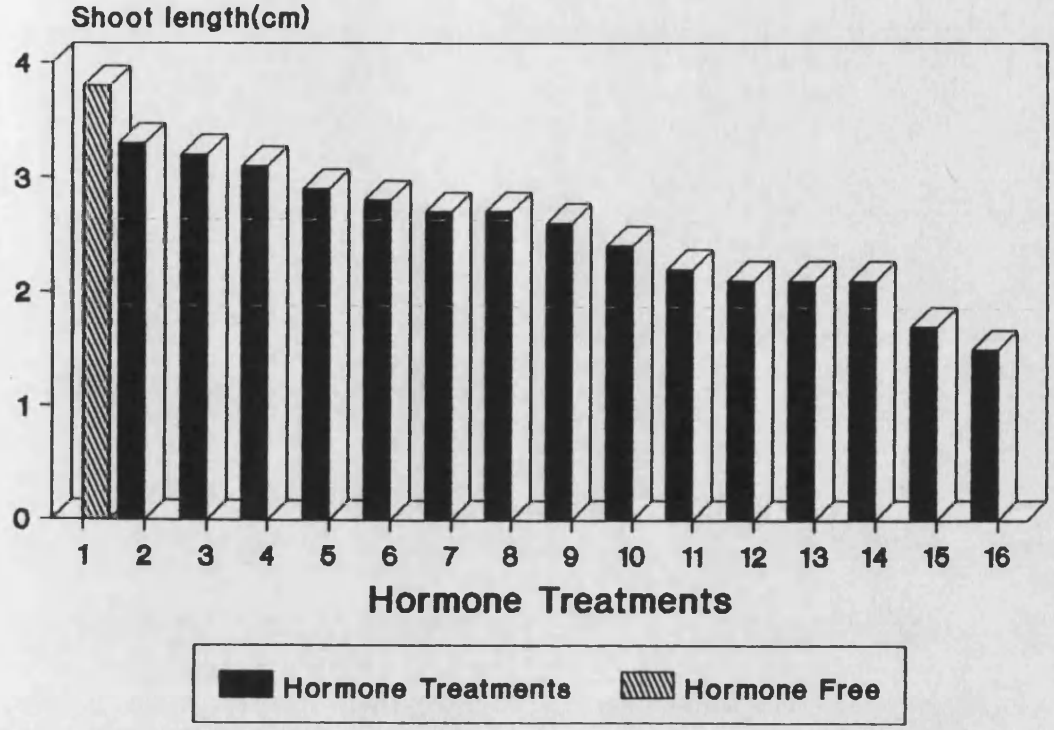
Fig 3: Effect of hormone treatments on propagule numbers in cv.Pepinex



Key
Treatments(μM):

1. 0.4 BA
2. 0.6 BA
3. 0.3 NAA+ 0.4 BA
4. 0.6 NAA+ 0.3 BA
5. 0.4 NAA+ 0.6 BA
6. 0.3 NAA
7. 0.6 NAA+ 0.4 BA
8. 0.4 NAA+0.4 BA
9. 0.6 NAA+ 0.6 BA
10. 0.3 NAA+ 0.6 BA
11. 0.4 NAA
12. 0.6 NAA
13. 0.3 NAA+ 0.3 BA
14. 0.3 BA
15. Hormone-Free
16. 0.4 NAA+ 0.3 BA

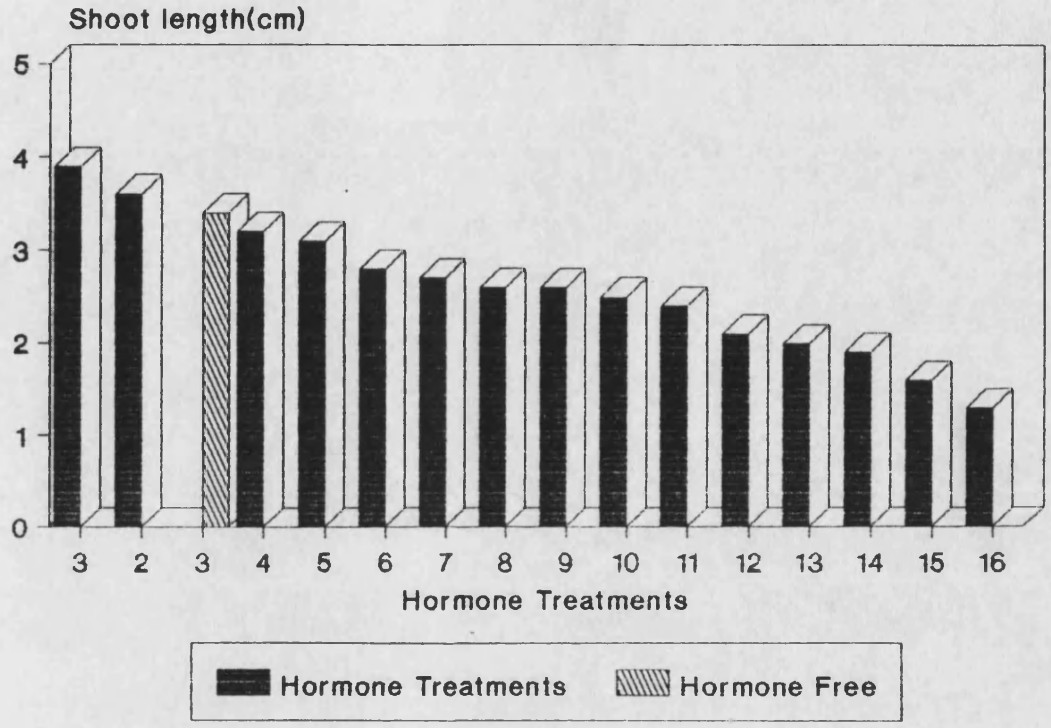
Fig 4.Effect of hormone treatments on shoot length in cv. Telegraph



Key
Treatments(μM):

1. Hormone-Free
2. 0.6 NAA+ 0.4 BA
3. 0.3 NAA+ 0.4 BA
4. 0.3 NAA+ 0.6 BA
5. 0.3 NAA
6. 0.3 NAA+ 0.3 BA
7. 0.6 BA
8. 0.6 NAA
9. 0.4 BA
10. 0.4 NAA+ 0.3 BA
11. 0.4 NAA
12. 0.3 BA
13. 0.4 NAA+ 0.6 BA
14. 0.4 NAA+ 0.4 BA
15. 0.6 NAA+ 0.6 BA
16. 0.6 NAA+ 0.3 BA

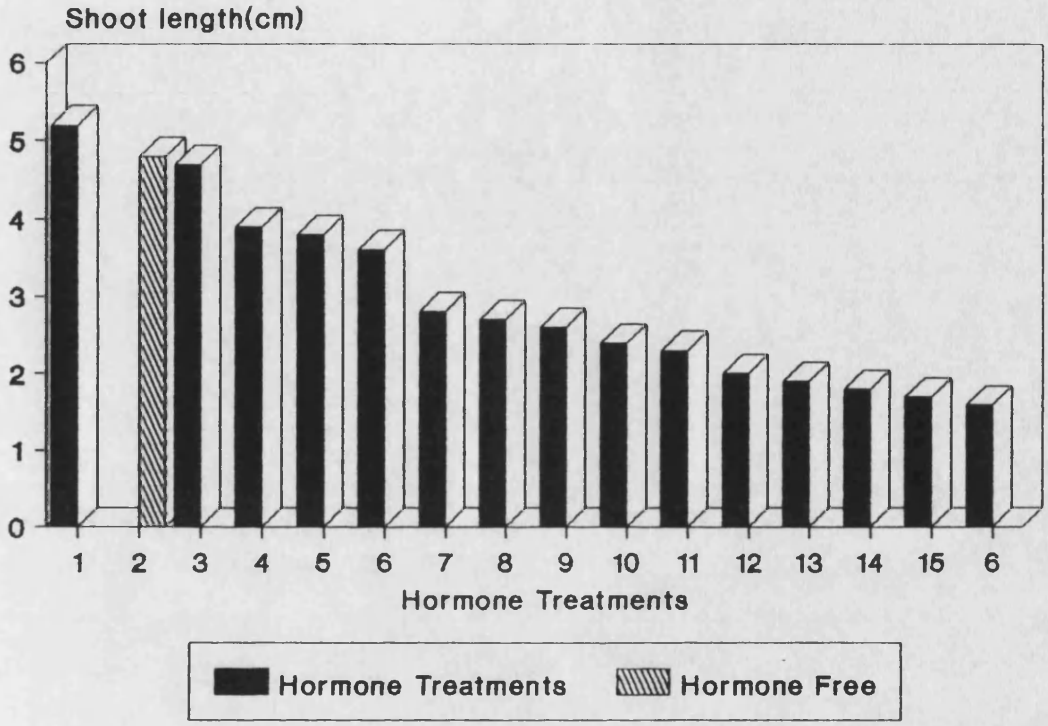
Fig.5. Effect of hormone treatments on shoot length in cv. Rebella



Key
Treatments (μM):

1. 0.6 BA
2. 0.3 NAA
3. Hormone-Free
4. 0.6 NAA
5. 0.3 NAA+ 0.6 BA
6. 0.4 NAA+0.3 BA
7. 0.6 NAA+ 0.4 BA
8. 0.4 BA
9. 0.6 NAA+ 0.3 BA
10. 0.3 NAA+ 0.3 BA
11. 0.6 NAA+ 0.6 BA
12. 0.4 NAA+ 0.4 BA
13. 0.3 BA
14. 0.4 NAA
15. 0.4 NAA+ 0.3 BA
16. 0.3 NAA+ 0.4 BA

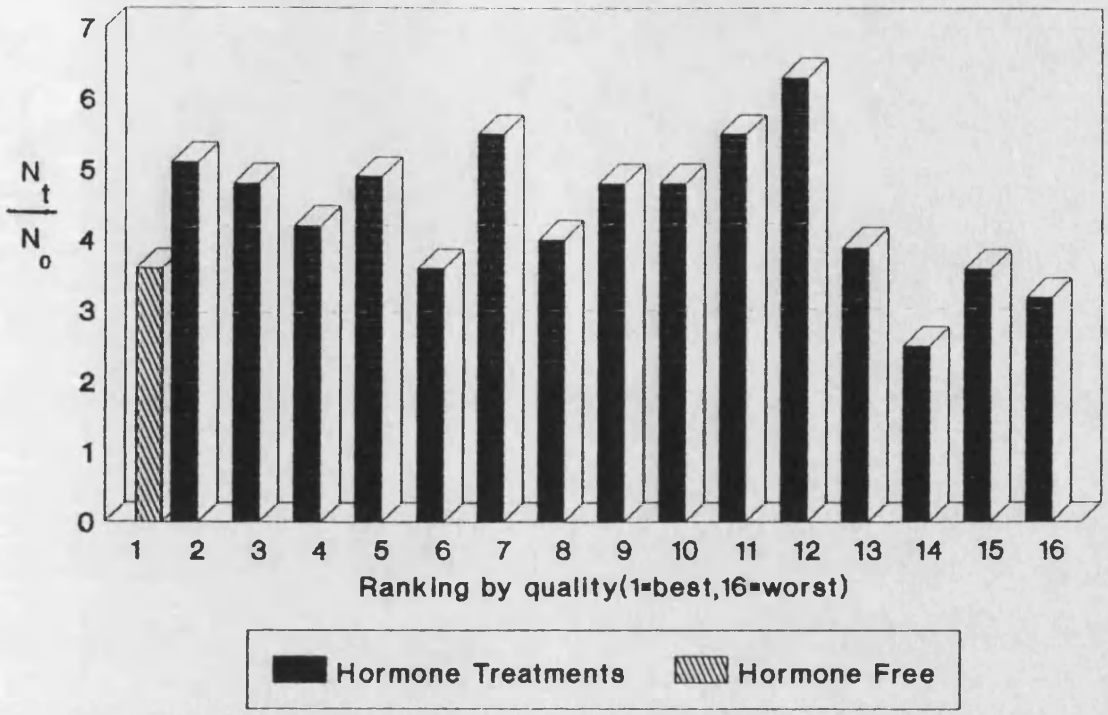
Fig.6. Effect of hormone treatments on shoot length in cv. Pepinex



Key
Treatments(μM):

1. 0.6 NAA
2. Hormone-Free
3. 0.6 NAA+ 0.3 BA
4. 0.6 NAA+ 0.4 BA
5. 0.6 BA
6. 0.3 NAA
7. 0.4 NAA+ 0.6 BA
8. 0.4 BA
9. 0.4 NAA
10. 0.6 NAA+ 0.6 BA
11. 0.3 NAA+ 0.6 BA
12. 0.3 NAA+ 0.4 BA
13. 0.3 NAA+ 0.3 BA
14. 0.3 BA
15. 0.4 NAA+ 0.4 BA
16. 0.4 NAA+ 0.3 BA

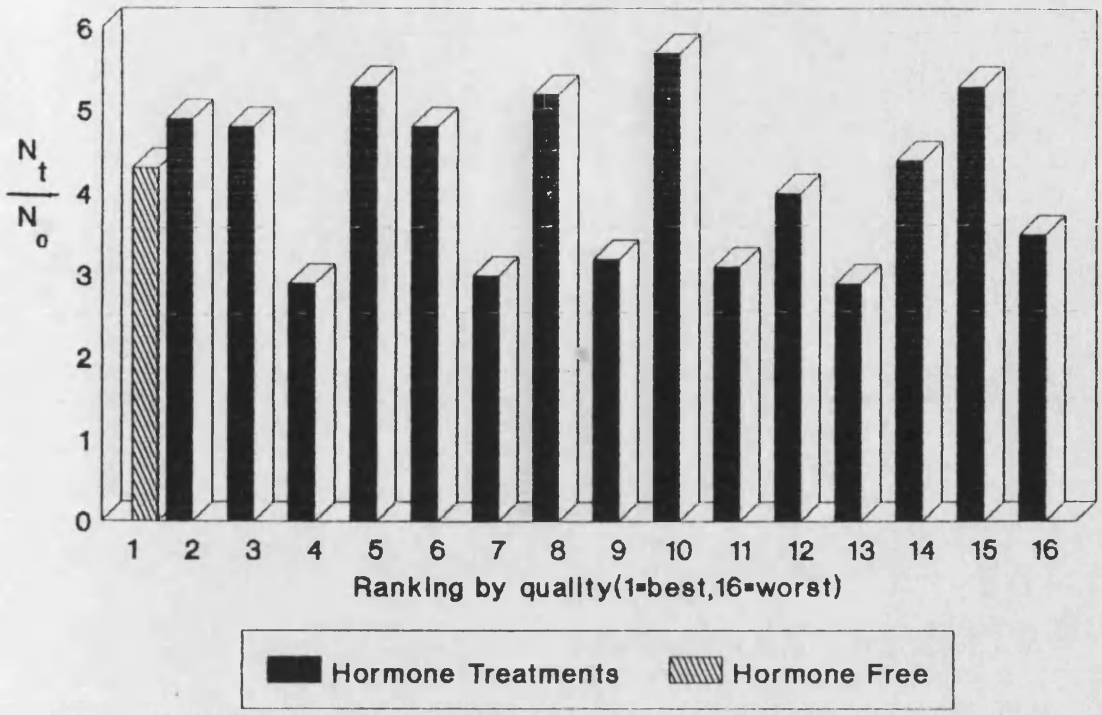
Fig.7. Effect of hormone treatments on
cv. Telegraph plantlet quality



Key
Treatments(μM):

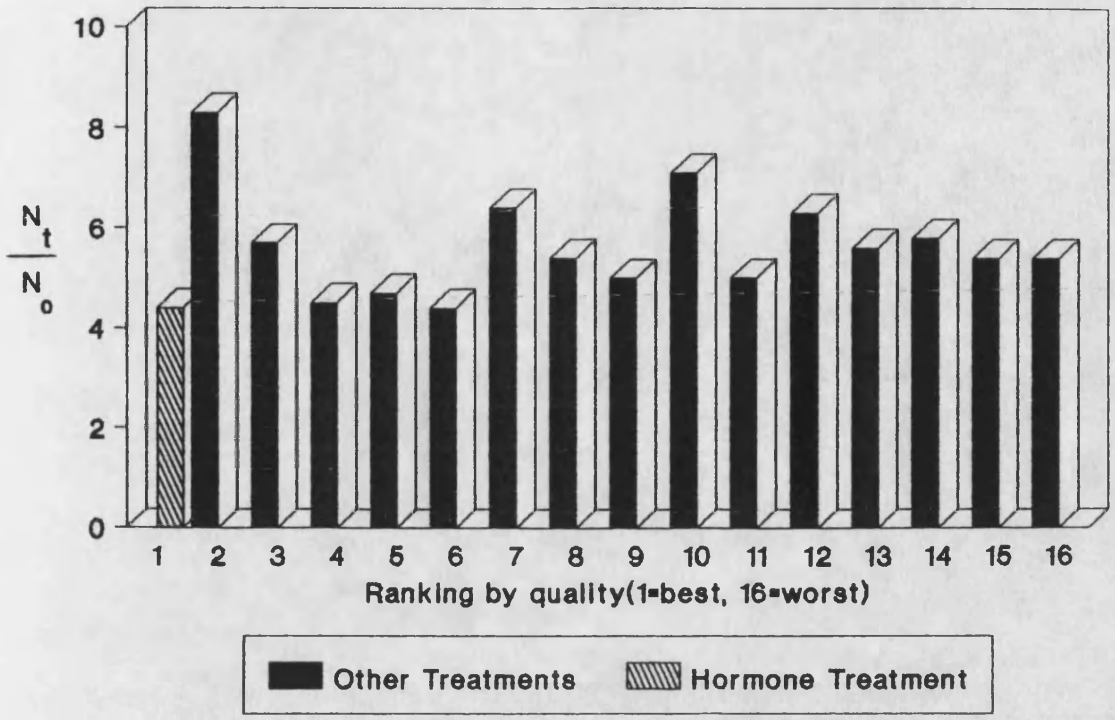
1. Hormone-free
2. 0.4 BA
3. 0.3 NAA+ 0.4 BA
4. 0.3 NAA+ 0.3 BA
5. 0.4 NAA+ 0.3 BA
6. 0.4 NAA+ 0.4 BA
7. 0.3 NAA
8. 0.4 NAA
9. 0.3 BA
10. 0.6 NAA+ 0.4 BA
11. 0.3 NAA+ 0.6 BA
12. 0.6 BA
13. 0.4 NAA+ 0.6 BA
14. 0.6 NAA
15. 0.6 NAA+ 0.6 BA
16. 0.6 NAA+ 0.3 BA

Fig. 8. Effect of hormone treatments on
cv. Rebella plantlet quality



Key
Treatments(μM):
1. Hormone-Free
2. 0.4 BA
3. 0.3 NAA+ 0.3 BA
4. 0.4 NAA+ 0.3 BA
5. 0.3 NAA
6. 0.3 BA
7. 0.4 NAA
8. 0.4 NAA+ 0.4 BA
9. 0.3 NAA+ 0.4 BA
10 0.6 BA
11. 0.6 NAA
12. 0.6 NAA+ 0.4 BA
13. 0.6 NAA+ 0.3 BA
14. 0.4 NAA+ 0.6 BA
15. 0.3 NAA+ 0.6 BA
16. 0.6 NAA+ 0.6 BA

Fig.9. Effect of hormone treatment on
cv. Pepinex plantlet quality



Key
Treatments(μM):

1. Hormone-Free
2. 0.4 BA
3. 0.3 NAA
4. 0.3 BA
5. 0.3 NAA+ 0.3 BA
6. 0.4 NAA+ 0.3 BA
7. 0.3 NAA+ 0.4 BA
8. 0.4 NAA+0.4 BA
9. 0.4 NAA
10. 0.6 BA
11. 0.6 NAA
12. 0.6 NAA+ 0.3 BA
13. 0.6 NAA+ 0.4 BA
14. 0.4 NAA+ 0.6 BA
15. 0.6 NAA+ 0.6 BA
16. 0.3 NAA+ 0.6 BA

On a hormone-free MS medium, unbranched shoot growth with an increase in the number of nodes (i.e. propagules) was observed in all cultured explants. The geotropic response of the plants on hormone free media was usually abnormal, but in other ways they looked healthier than the hormone treated plantlets, except those obtained on 0.3 μM NAA and 0.4 μM BA. Plantlets on hormone free media had very long internodes.

Addition of BA to the basal medium in the range of 0.3-0.6 μM resulted in multiple shoots per explant. The shoot proliferation rate increased with increasing of BA concentration. Taking the quality of shoots produced into account, the highest number of vigorous shoots was obtained on 0.4 μM BA. The survival rate of shoot tip explants ranged from 92 to 100%. Increasing the BA concentration from 0.4 μM to 0.6 μM decreased the vigour of shoots. Vitrification was observed at both 0.3 and 0.6 μM BA concentrations but not at 0.4 μM BA.

An increase in propagule numbers was also obtained on the media containing NAA without BA. The highest propagation rate observed in such media was obtained on 0.3 μM NAA (Table 2).

Of the various combinations of NAA and BA tested, low concentrations of both hormones tended to give higher shoot propagule ratios than the high ones.

Tables 2 to 4 also show the hormone treatments ranked according to the quality of propagules produced.

Factors taken into account for ranking treatments according to propagule quality were the amount of unwanted callus produced, the length of internodes and the general vigor of the plantlets.

Data on the lengths of main shoots are also shown in Tables 2 to 4. In cultivar Telegraph there were no significant differences between shoot lengths on the various hormones; in Pepinex and Rebella, statistically significant differences between some treatments were found, but these differences did not seem to be related to the hormone treatments.

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Table 5. The effect of hormone treatments on the rooting response (% cultures having developed roots in 32 days) HF= Hormone free medium. concs= full strength.

Cult/Treatment	HF	BA	NAA	NAA+ BA
Telegraph	90	55	55	45
Rebella	100	79	75	52
Pepinex	100	77	71	51

Table 6. The effect of different hormone treatments on the extent of root formation. (Mean scores on an empirical scale from 0 to 5). HF= hormone free medium. concs= full strength.

Cult/Treatment	HF	BA	NAA	NAA+BA
Telegraph	2.3	2.2	2.1	1.8
Rebella	2.1	2.9	2.9	1.2
Pepinex	2.4	2.7	2.7	2.0

The summary of the results for callusing is shown in the text in Tables 7 to 8, and in details in Tables 9 to 11 in Appendix 2.

Callusing was observed at the bases of the explants.

As expected, hormones produced callusing (Table 7) although cv Telegraph produced callus on 50% of the explants even in the hormone free medium. BA and NAA were approximately equally effective, and have induced callus on about 90% of the explants. The same happened in the presence of the two hormones applied in combination. The extent of callusing shows a similar pattern, with significant amounts of callus formation only on media containing hormones; the type of hormone was not important, and the effect of combining the two types of hormones was less than additive.

Table 7. The effect of hormone treatments on callusing, (% of cultures having developed basal callus in 32 days). HF= hormone free medium. concs= full strength.

Cult/Treatment	HF	BA	NAA	NAA+BA
Telegraph	50	87	90	96
Rebella	9	97	92	100
Pepinex	0	87	93	93

Table 8. Effect of hormone treatments on the extent of callusing after 32 days in culture (scored on an ascending scale from 0 to 5). HF= hormone free medium. concs= full strength.

Cult/Treatment	HF	BA	NAA	NAA+BA
Telegraph	0.4	3.5	4.5	4.7
Rebella	0.5	3.2	3.5	3.6
Pepinex	0.0	4.1	3.9	4.5

Based on the combined observations of propagule numbers and quality, and taking data on all 3 experimental cultivars as shown in Figs 1 to 9 into account, it was decided to use two hormone treatments, 0.3 μM NAA and 0.4 μM BA in further experiments for optimizing the protocol for clonal propagation.

Experiment 2: Choice of explants.

a. Three passages of 3 weeks each.

As described in Experiment 1. the two hormone concentrations chosen for further experimentation on the grounds of the numbers and quality of shoots produced in the three cultivars tested were 0.3 µM NAA and 0.4 µM BA. Next, it was necessary to see whether the choice of explants (i.e. shoot tips or nodes) was important or not.

Shoot tips and two uppermost nodes (nodes 1 and 2) all 3 to 5 mm long, were therefore chosen as explants. The explants were obtained from 25 day old in vitro grown plants and placed on the selected hormone treatments, usually three explants per jar. 9 to 15 explants were used per treatment. The same cultivars i.e. Telegraph, Rebella and Pepinex were used as before.

The explants were maintained at 25±1°C with a 16 hr photoperiod and at 2.9 Wm^{-2} light intensity in the growth room.

At the end of 3 weeks, the shoot tips and the nodes were counted and explants were selected at random from shoot tips two uppermost nodes and were recultured for a second passage. The rest of the material was discarded. At the end of the second passage (i.e. another 3 weeks) again propagules were counted and some explants were used for a third passage as above.

From the numbers of explants at various times, the cumulative values of $\frac{N_t}{N_0}$ were plotted against time, the propagule doubling time were calculated from the expression $t_d = \frac{\ln 2}{K}$ as explained in chapter 2.

As shown in Table 12, the propagule doubling times derived were almost invariably lower for cultures grown on BA than on NAA.

The quality of plantlets grown on media containing BA was also better than that of those grown on NAA.

Table 12. Propagule doubling times over 3 passages of 3 weeks each (days).

Cult. and explant type / Hormone Trt	Telegraph			Rebella			Pepinex		
	Shoot tip	Node 1	Node 2	Shoot tip	Node 1	Node 2	Shoot tip	Node 1	Node 2
0.3 μ M NAA	14.7	25.6	12.1	17.7	13.5	12.3	16.4	17.3	13.3
0.4 μ M BA	9.5	11.3	12.5	10.0	10.3	9.7	10.5	9.5	9.5

As shown by Figs 10 to 27 in Appendix 2 the proliferation rate curves turned out to be statistically straight lines, except for Fig 23 where the ratio at the end of the first passage after 21 days was anomalously low. Propagule doubling times could therefore be calculated unambiguously from these curves.

Comparison of propagule doubling times in Table 12 shows that the main factor determinig the proliferation rate is the hormone treatment. Genotype seemed to have only a minor effect and the choice of explant was not significant for cultures containing BA, but for the cultures containing NAA it was significant.

An unexpected feature of these experiments was the observation that in some cases transition to flowering occurred within a 3 week culture passage. The cultures affected were some of those in their third passage; 20 to 30% of plantlets derived from shoot tips or first nodes of cv. Telegraph on NAA medium showed this response.

Qualitatively the flowering was not a major development but, because of the general importance of the vegetative to reproductive phase transition and because of the potential practical difficulties that such premature transition could cause in a large scale clonal propagation scheme, it was decided to investigate the phenomenon in further experiments.

b. Three passages of 4 weeks each.

As mentioned above, the transition to flowering in the previous experiment it has been observed in only a small number of cultures. In view of the importance of this phenomenon,

it was decided to establish the observations more firmly before embarking on a longer and more detailed examination. The previous experiment (Exp.2) was therefore repeated, but with 3 culture passages of 4 weeks's duration each, instead of the previous 3 weeks's.

The shoot proliferation rate curves are shown in figs 28-39 (Appendix 2); and the propagule doubling times derived from them are summarized in Table 13. As shown by comparison with Table 12, the propagule doubling times derived from the experiment based on 4 week culture passages are either similar or slightly longer than those based on 3 week passages, as would be expected.

Table.13. Propagule doubling times over 3 passages of 4 weeks each (days).

Cult. and explant type / Hormone Trt	Telegraph			Rebella			Pepinex		
	Shoot tip	Node 1	Node 2	Shoot tip	Node 1	Node 2	Shoot tip	Node 1	Node 2
0.3 µ M NAA	*	*	*	14.4	16.0	12.5	*	*	*
0.4 µ M BA	12.0	12.3	10.8	13.0	11.1	11.0	11.0	11.7	10.3

*= Extensive transition to flowering have occurred in these cultures, hence data for propagule doubling times could not be calculated.

Flowering was a more common feature of this experiment than it was on the previous one (Figs 40-44).

Fig 40. Transition to flowering during 3 culture passages of 4 weeks each on 0.3 μ M NAA, cv. Telegraph

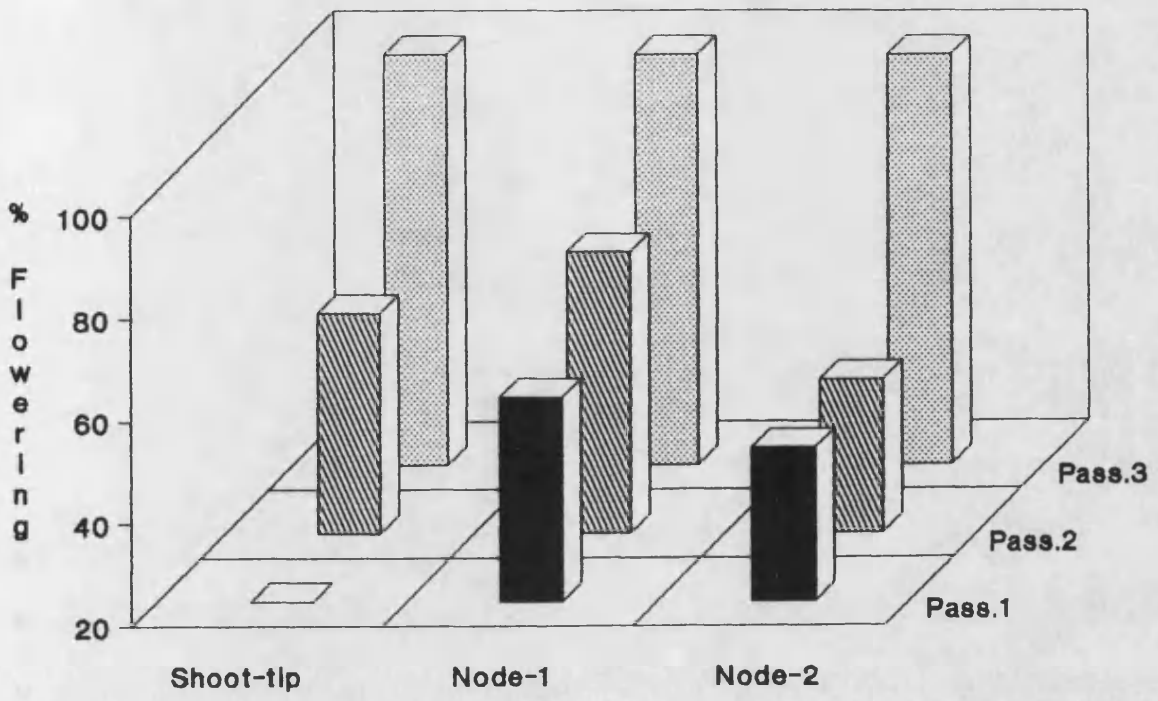


Fig 41. Transition to flowering during 3 culture passages of 4 weeks each on 0.4 μ M BA, cv. Telegraph

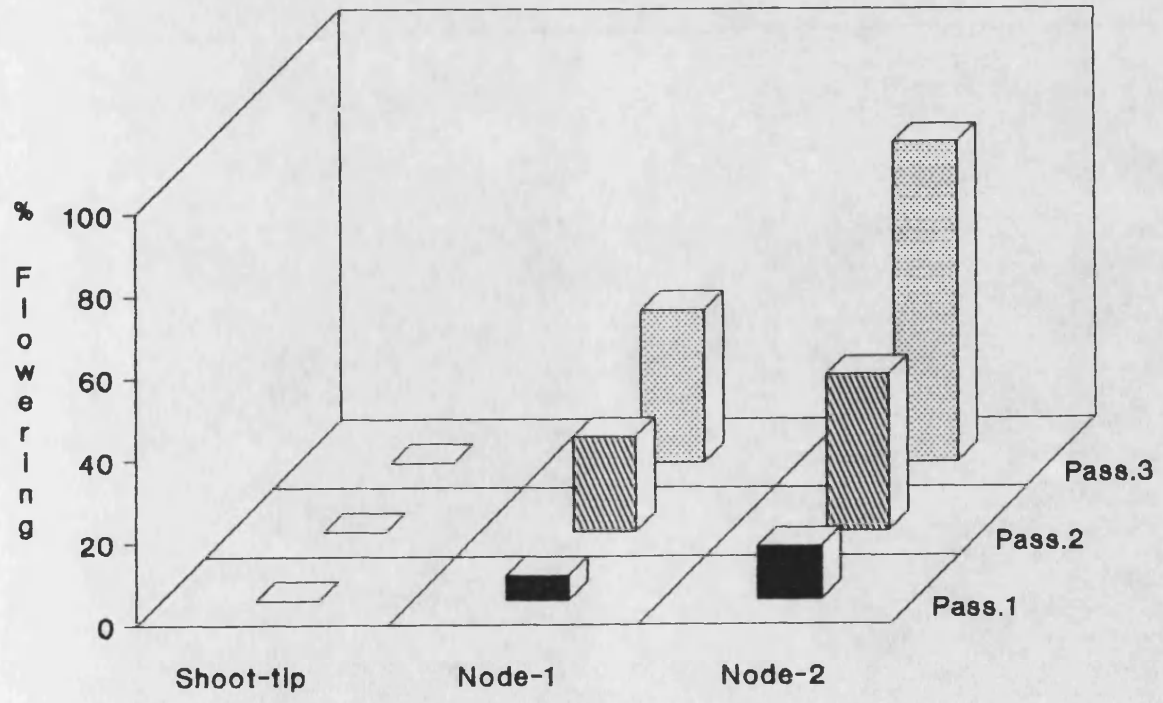


Fig 42.Transition to flowering during
3 culture passages of 4 weeks each
on 0.3 μ M NAA, cv. Rebella

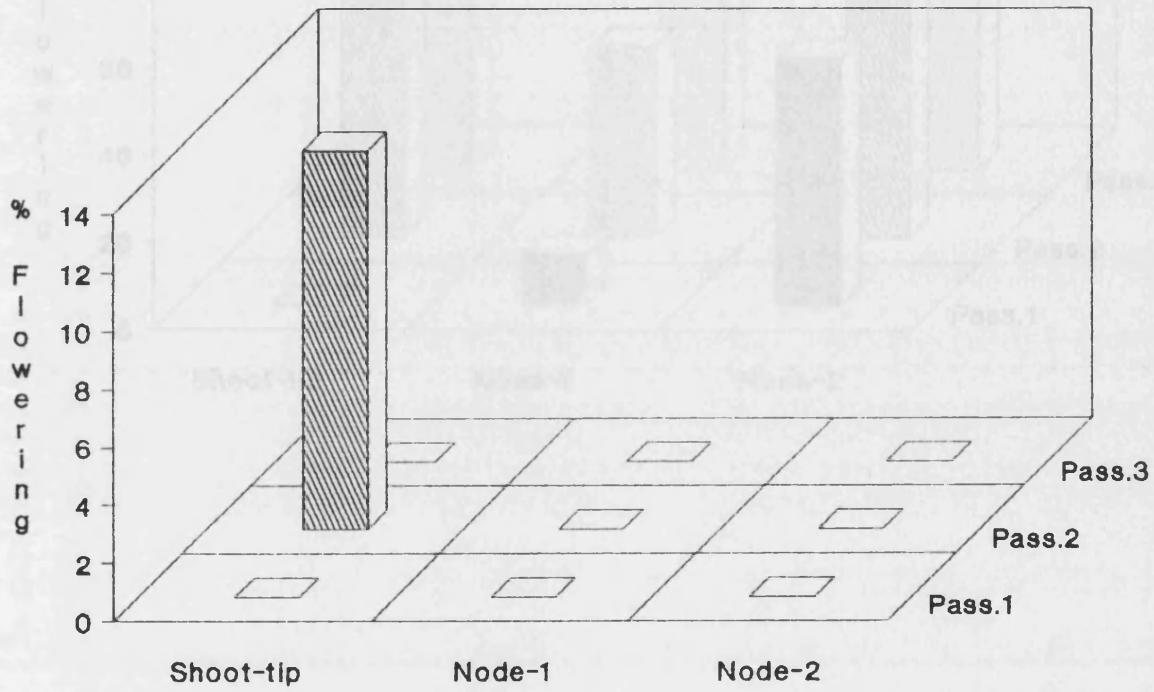


Fig 43. Transition to flowering during
3 culture passages of 4 weeks each
on 0.3 μM NAA, cv. Pepinex

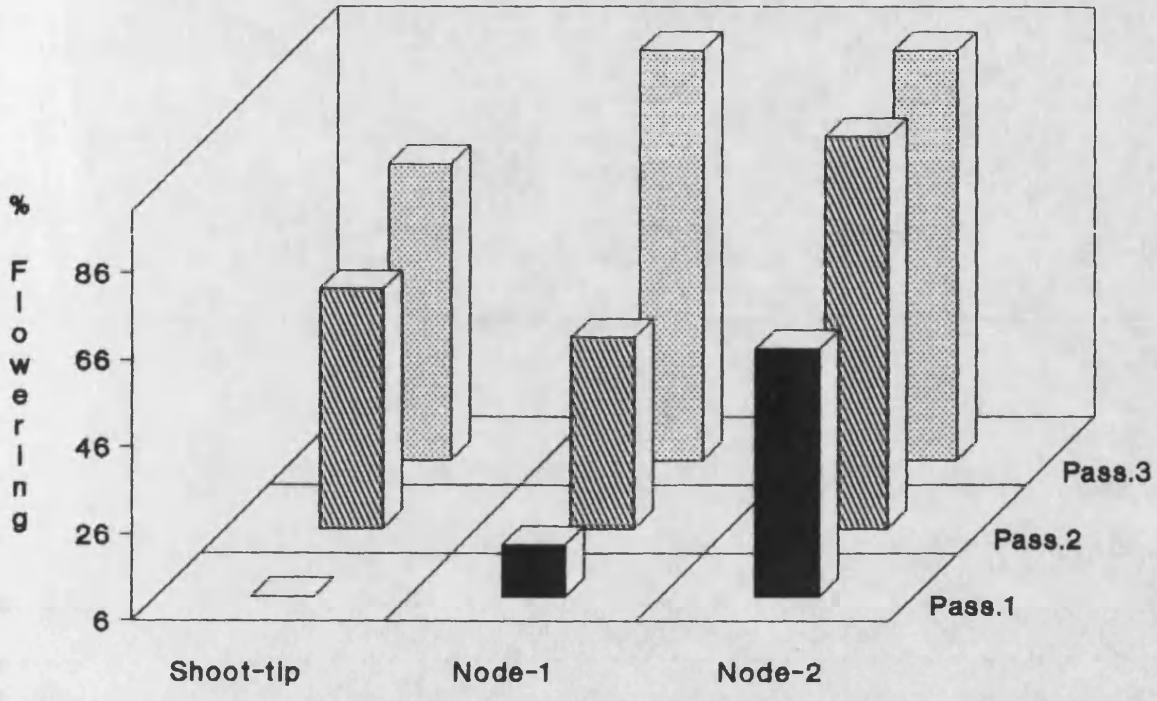
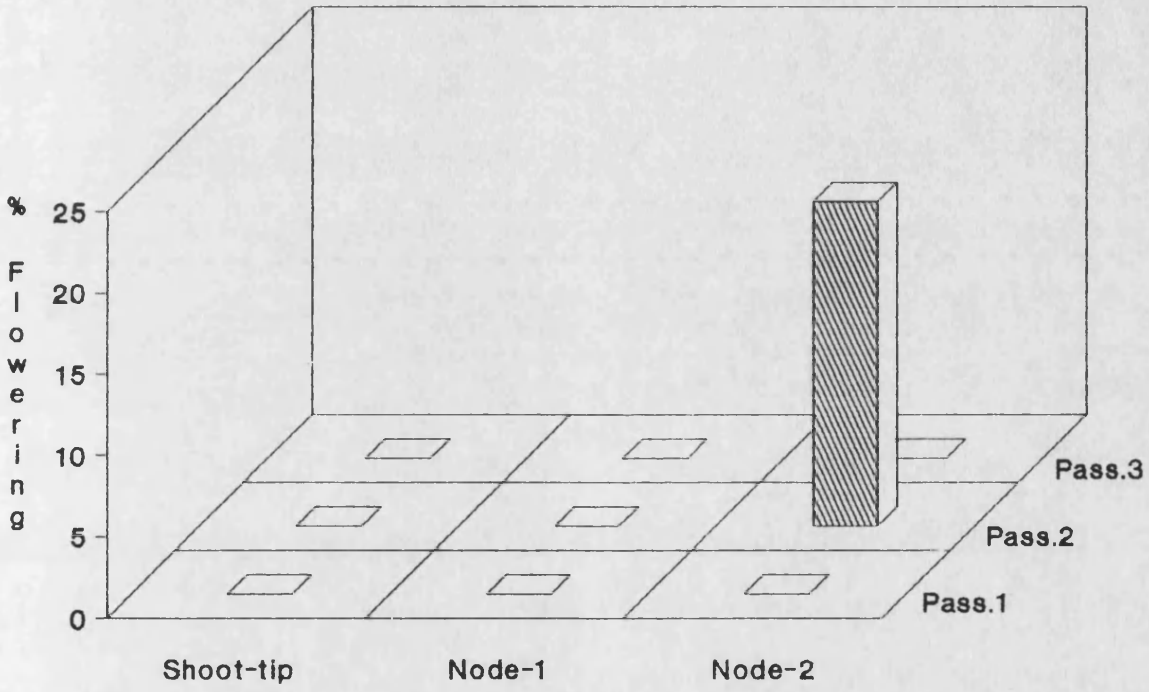


Fig 44. Transition to flowering during
3 culture passages of 4 weeks each
on 0.4μ M BA, cv. Pepinex



Flowering was observed in cvs. Telegraph and Pepinex in cultures derived from all explants and in cv. Rebella only in cultures arising from shoot tips. Flowering was most pronounced on the cultures growing on NAA containing media, but cultures from first and second nodes of cv. Telegraph flowered also on media containing BA.

The transition to flowering was gradual, but it has affected nearly 100% of the cultures of cultivars Telegraph and Pepinex on NAA media. In general transition to flowering tended to be more pronounced in node-derived cultures, than in shoot-tip derived ones.

In the context of developing a protocol for continuous clonal propagation applicable to all cultivars, transition to flowering is undesirable. It was therefore decided to investigate the effect of temperature on the transition to flowering over a range of 10°C, from 20°C to 30°C.

Experiment 3. Effect of temperature.

a) Choice of suitable temperature for optimal shoot proliferation.

The three experimental temperatures used in this experiment were 20, 25 and 30°C.

In previous experiments, shoot tips tended to flower less than nodes, therefore shoot tips were used as explants in this experiment.

Explants 3 to 5 mm long, were obtained from 13 day old *in vitro* grown seedlings.

All the explants were cultured at the specified temperatures for 33 days . Photoperiod was 16 hrs and light intensity was 14 W m^{-2} . Each treatment was replicated 9 to 11 times. The explants were cultured in 175 ml glass jars and 2 to 3 explants were cultured per jar.

After 33 days, the number of propagules(shoot tips and nodes) were counted and used for calculation of $\frac{N_t}{N_0}$. The shoot length were also measured .

The results are shown in Figs 45 and 46, and in Tables 14 and 15, in Appendix 2. Propagule numbers were not affected significantly by either temperature, or genotype (Fig 45), although in general, the propagation ratio tended to increase with temperature. Shoot length increased significantly with temperature for all three cultivars (Fig 46). Flowering was observed in 1 out 11 cultures of cv. Pepinex at 25°C, and in 2 cultures each out of 9 and 12 cultures at 20 and 25°C respectively of cv. Telegraph.

b. Choice of suitable passage duration at 30°C.

Based on results obtained previously, 30°C appeared to provide the most favourable conditions for continued clonal propagation and the object of this experiment was to compare the proliferation rates obtained over a 3 months period, subculturing either every 15, or every 30 days. The results are shown in Tables 16 and in Figs 45 to 50 in Appendix, 2. In all cultivars , propagule doubling time was shorter on 15 day culture passages than on 30 day ones . As regards propagule quality plantlets obtained from 15 day passages were better than those from 30 day passages (Plate 12). Some callusing was also observed at the base of the plantlets subcultured at 30 day intervals, but this has not a problem with those subcultured every 15 days. On balance, subculturing at 15 day intervals was found to be more appropriate than once every 30 days.

Table 16. Propagule doubling times over 3 months, using 15 and 30 day subculture intervals, on 0.35 µM BA at 30°C. Explant: shoot tip

Intervals (days)	15	30
Telegraph	6.9	12.8
Rebella	6.6	9.9
Pepinex	7.0	11.0

Establishment of plantlets in compost.

Establishment of tissue cultured plantlets is the final stage in the process of micropropagation and is of vital importance for production of healthy, high quality material and also for in vivo assessment (Langford, 1987).

The success of any micropropagation system can only be affectively measured by the number of plants which are succssfully transferred from tissue culture vessels to soil conditions.

To assess the success of our clonal propagation techniques, at the end of one of our experiments, we removed the plantlets produced from the proliferation medium and transferred them to No 9 plastic pots (as explained in chapter 2). After two months culture in glasshouse the plants were compared with plants were obtained from the seeds.

There was no variability in plants grown in vitro and more than 95% of the plants established and produced fruits in the same quality as those grown from seed (plate, 1 & 2).

Plate 1. In vitro grown plantlets 10 days after transfer to compost.

Plate 2. Plants from in vitro culture 2 months after establishment in compost.

DISCUSSION

The only report in the literature concerned with the clonal propagation of cucumber appears to be that of Handley & Chambliss (1979). These authors used axillary bud explants taken from plants of a single genotype "Carolina" grown in vivo. The explants were cultured for 4 weeks on MS medium with and without hormones; their results, in terms of the percentage of explants regenerated into plantlets, are shown in Table,17.

Their results show that axillary buds do not develop into plantlets on a hormone-free medium; but, interestingly enough, a significant number of plants could be regenerated using either NAA, or kinetin only. The highest percentage regeneration was 42% with kinetin only, and 67% on NAA as the sole exogenous hormone. The highest percentage regeneration (83%) occurred on a medium containing both NAA and kinetin at 0.1 ppm. The quality of the plantlets produced was also optimal under these conditions.

In Handley & Chambliss's experiments, complete plantlets, including root systems, were regenerated; the root systems are the likely source of cytokinin in the cultures to which only NAA has been added as a hormone. The paper provides no more than a starting point for the development of a protocol for the clonal propagation of cucumber, because the data were based on one cultivar, one type of explant and on a single culture passage only.

Table 17. Percentage of plantlets produced from axillary bud explants after 4 weeks of culture on different hormone treatments (from Handley and Chambliss, 1979). n=12

NAA kinetin (ppm)	0.0	0.02	0.1	0.5
0.0	0	0	67	0
0.02	0	0	58	0
0.1	0	8	83	0
0.5	17	0	17	0
1.0	42	33	0	0
2.0	17	0	0	0
5.0	8	0	0	0
10.0	0	0	0	0
15.0	0	0	0	0
20.0	0	0	0	0

The present work was carried out using three cultivars throughout: Telegraph, Rebella and Pepinex.

In the first experiment, designed to find the optimum hormone regime, shoot tips and nodes with expanded internodes (>0.5 cm) were counted as propagules. The results show (Figs 1-3 and Tables 2-4, Appendix 2) that with all three cultivars, it was possible to obtain high propagation rates on either BA or NAA containing media, or even on the hormone-free ones. As shown in plates 3-5, the characteristic feature of the plantlets regenerated on the hormone-free media are the long internodes.

The BA only treatments have produced multiple shoots; this was most obvious for cvs. Rebella and Pepinex (plates, 4- 5). in addition, plantlets regenerated on such media also had well developed root systems; root development has not been suppressed even at 0.6μM BA concentration (plate 6). On media containing NAA only, optimum shoot development occurred at a concentration of 0.3 μM; above this, shoot development was increasingly inhibited. The same trend has been observed for root development. The response to exogenous hormones was cultivar dependent; Pepinex responded well to high concentrations (0.6μM) of either BA or NAA (plate 6) while the growth of other cultivars was inhibited at these hormone concentrations. The combined application of both hormones was usually inhibitory; it appeared almost as if the effect of the two types of hormones on growth was additive. The occurrence of basal callus was minimal in hormone-free media, and increased with increasing total hormone concentrations.

Taking into account both the propagation rate and the quality of shoots produced, the 0.3μM NAA and the 0.4μM BA treatments were judged to the most promising, in spite of a small amount of basal callus development shown by such cultures . It is true

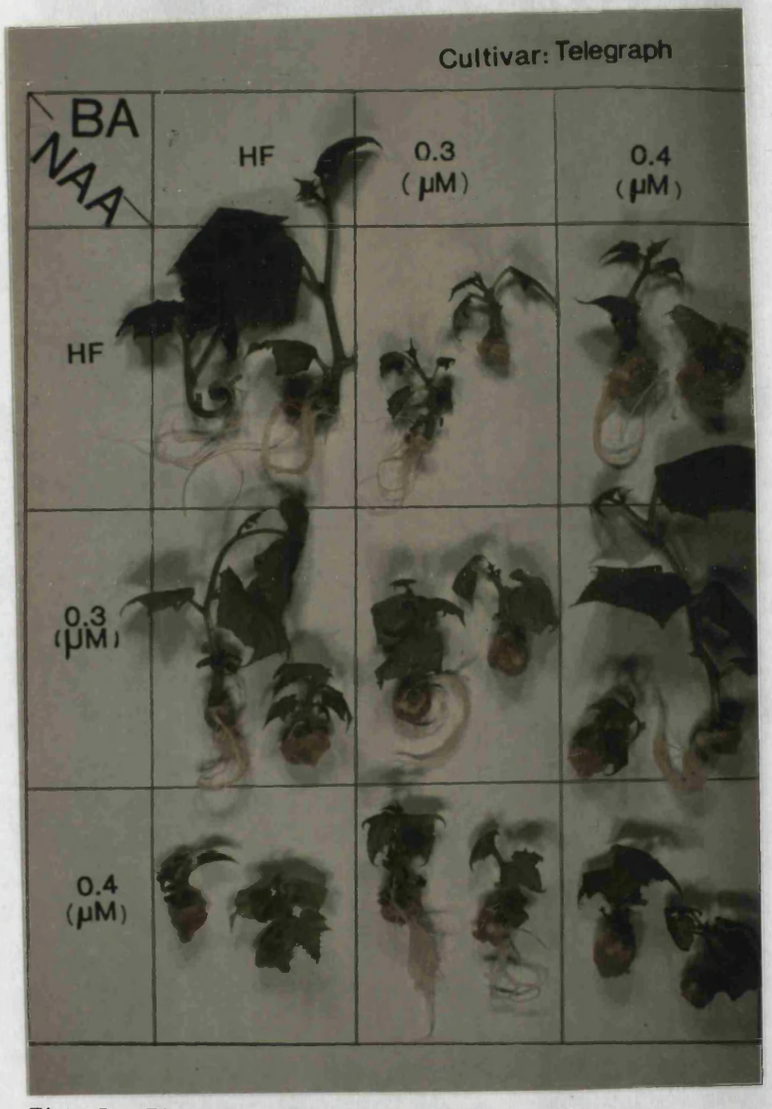


Plate 3 . Plantlets regenerated on the different media, cv. Telegraph. Note the long internodes on hormone-free medium. x 0.5

Plate 4. Plantlets regenerated on the different media, cv. Rebella. Note the multiple shoot production on 0.4 μM BA. x 0.5

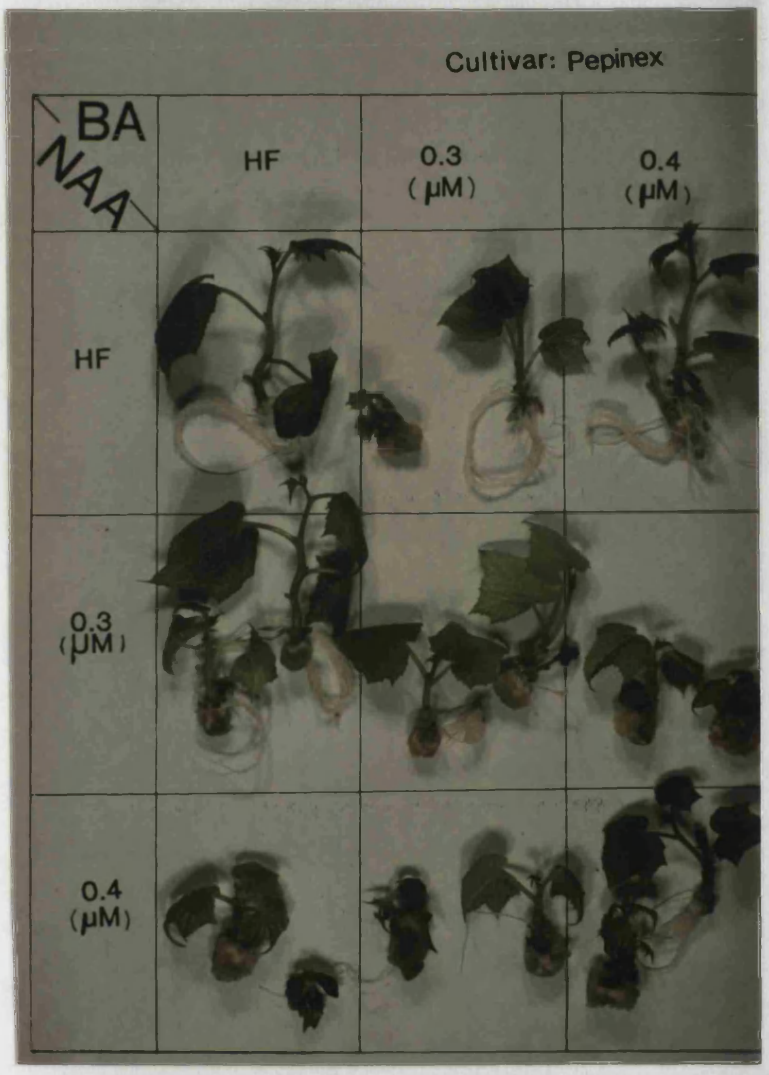


Plate 5. Plantlets regenerated on different thei media, cv. Pepinex. Note the multiple shoot production on 0.4 μM BA. x 0.5

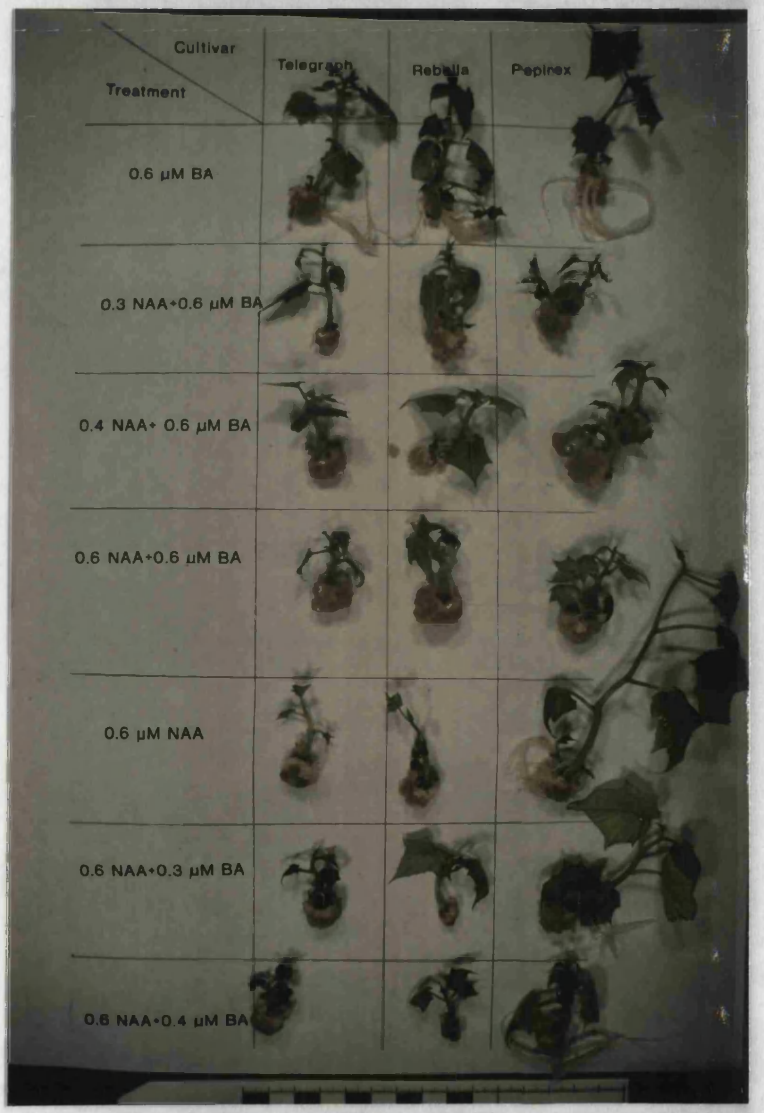


Plate 6 . Plantlets regenerated on the different media, and in various cultivars. Note the root initiation om media containing high BA concentration.

x 0.5

that good quality shoots with reasonable propagation rates could also be obtained on hormone-free media, but since such media are useful only for shoot tips but not for nodal explants (Table 17), they have not been used in further experiments for the development of a clonal propagation protocol.

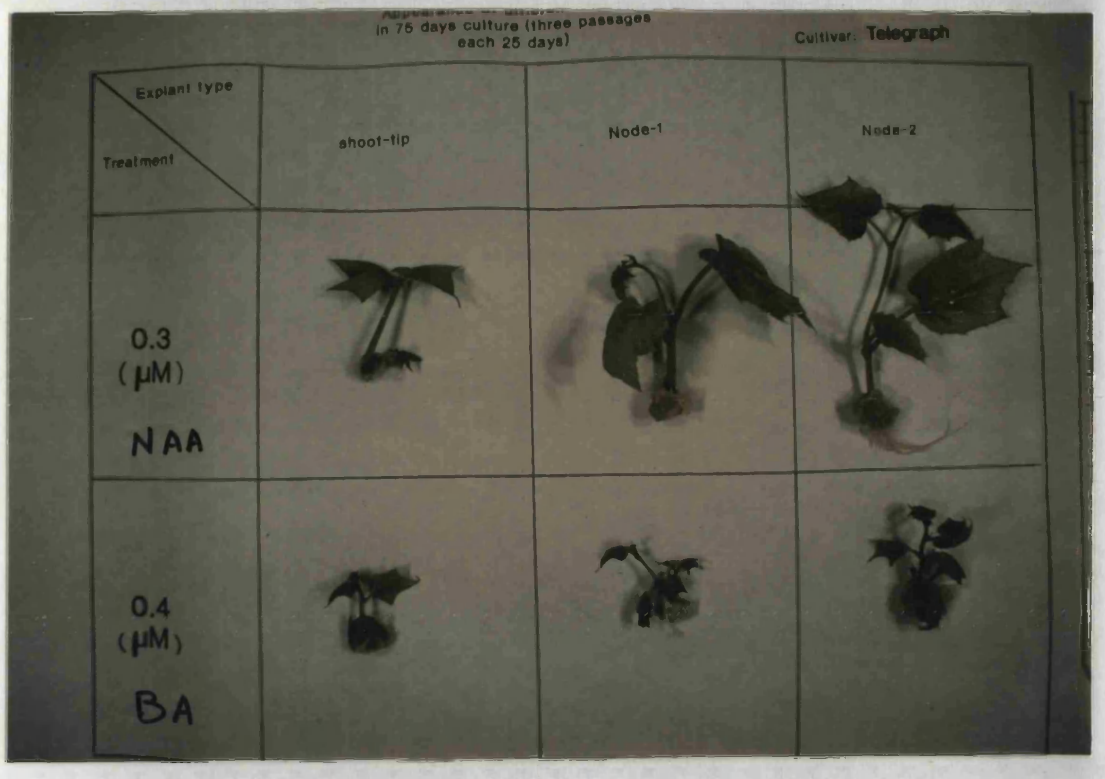
In the next experiment (Exp.2a), shoot tips and the uppermost two nodes were compared as explants on the two media selected previously (0.3 µM NAA and 0.4 µM BA). These experiments were carried out over 3 culture passages and the results were expressed as propagule doubling times; a low propagule doubling time implies a rapid propagation rate.

The results (Table 12) have shown that, with BA as the exogenous hormone, the propagation rate is faster than with NAA. This result is not surprising; cytokinin application results is the development of multiple shoots, whereas the auxin produces single shoots only, with expanded internodes (Plates 7-8). With cytokinin as the added hormone, the propagule doubling times obtained from all 3 types of explant were substantially the same. On the NAA medium, the propagule doubling times increased in the order Node 2 < Shoot tip < Node 1 ; the same trend has again been observed later in Exp.2b(Table 13) with cultivar Rebella. The consistently faster growths of plantlets obtained from node 2(Plates 7,8 & 9) explants compared with node 1 derived ones is particularly obvious. It would be tempting to ascribe such differences in growth rates to variations in endogenous hormone levels, but such measurements have not been carried out.

Transition to flowering is unusual in cultures maintained for clonal propagation purposes. Such transition was actually observed during the third passage of cv. Telegraph on NAA medium (Plate, 10).

Plate 7 (Top). Single and multiple shoot regeneration on different hormone type, cv. Telegraph in 25 day culture passage.

Plate 8 (Bottom). Effect of hormone and explant type on internode expansion, cv. Rebella.


in 75 days culture (three passages each 25 days)
Cultivar: Telegraph
Explant type
Treatment
shoot-tip
Node-1
Node-2
0.3 (μM) NAA
0.4 (μM) BA

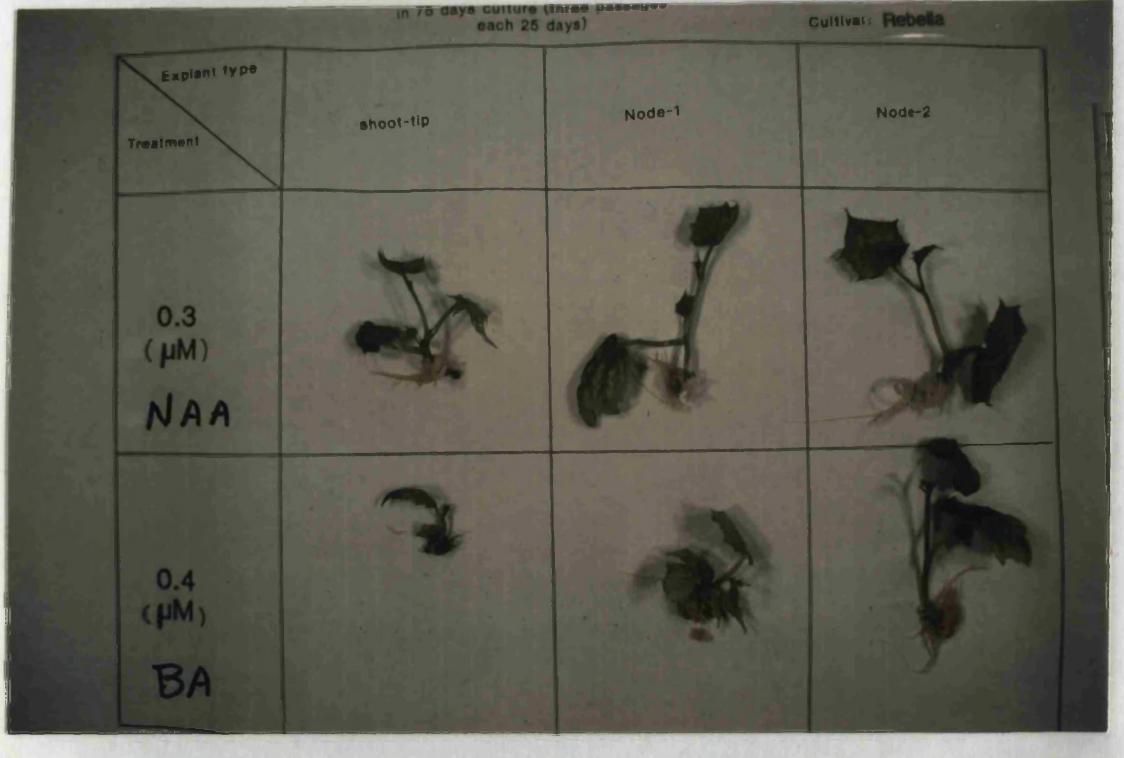
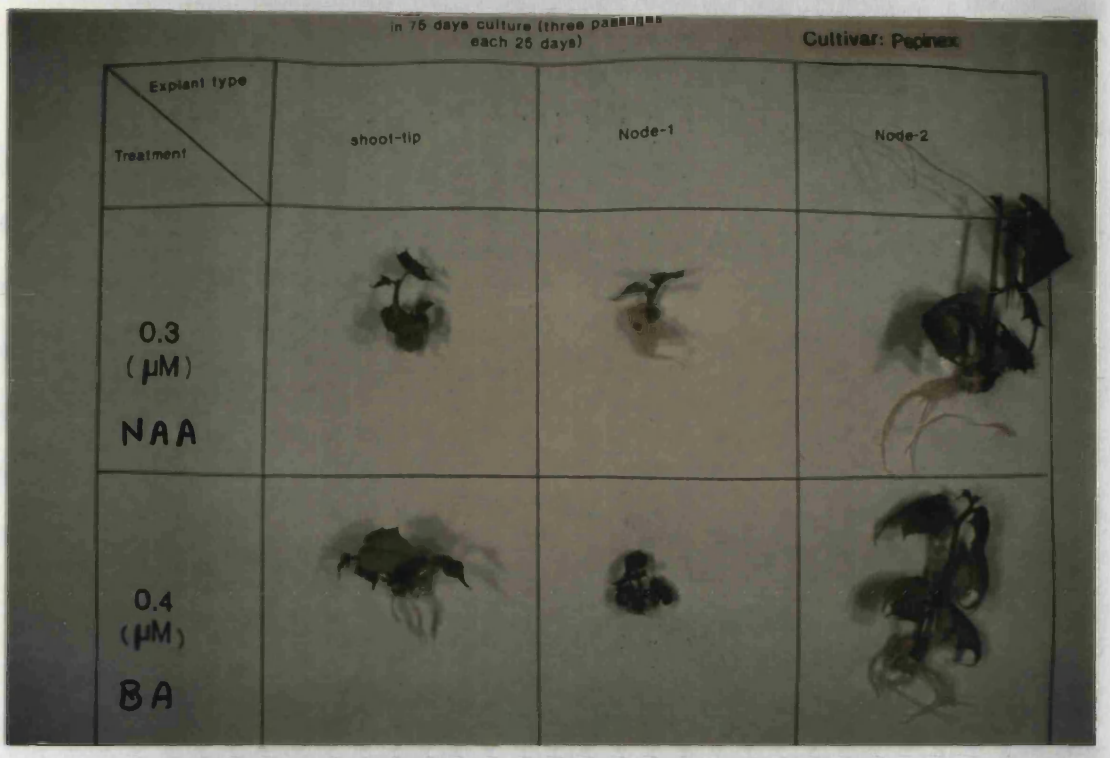
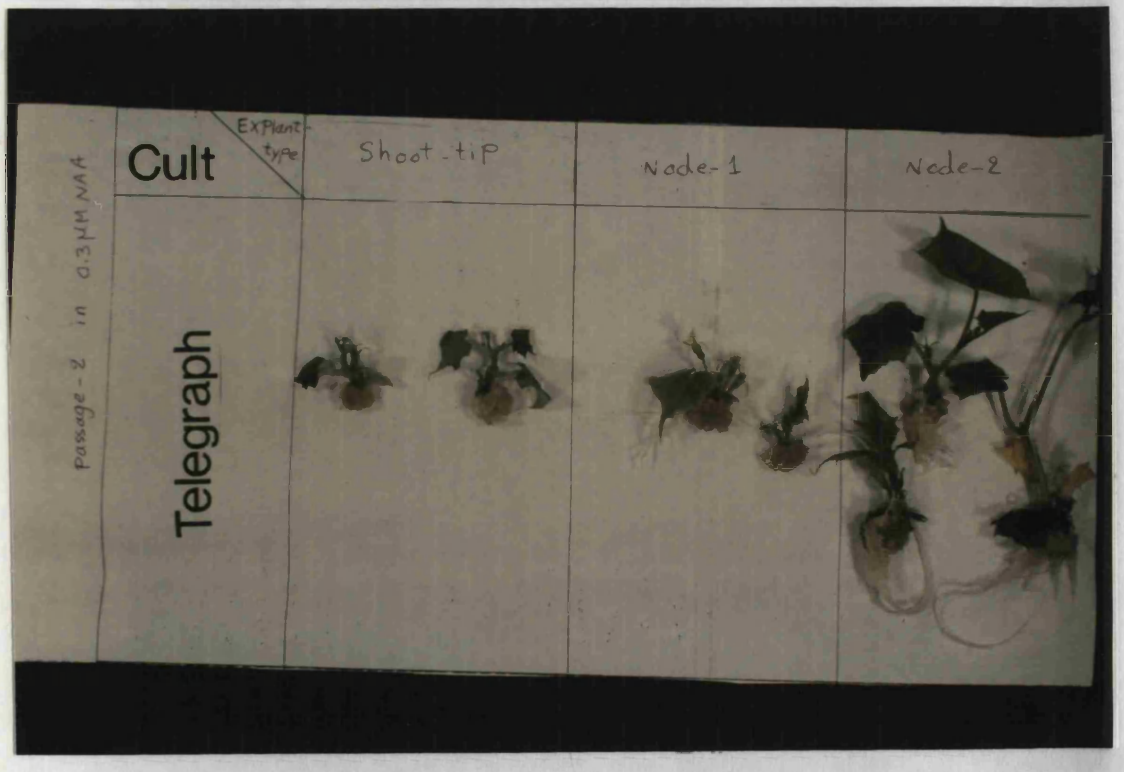

each 25 days)
Cultivar: Rebella
Explant type
Treatment
shoot-tip
Node-1
Node-2
0.3 (μM) NAA
0.4 (μM) BA

Plate 9 (Top). Single and multiple shoot regeneration on different hormone and explant type, cv. Pepinex in 25 day culture passage. x 0.5

Plate 10 (Bottom). Transition to reproductive stage on the media containing 0.3 μM NAA, cv. Telegraph. x 0.5


in 75 days culture (three pa
each 25 days)
Cultivar: Pepinex
Explant type
Treatment
shoot-tip
Node-1
Node-2
0.3
(μM)
NAA
0.4
(μM)
BA


Passage-2 in 0.3μM NAA
Cult
Explant-type
Shoot-tip
Node-1
Node-2
Telegraph

The phase transition has affected plantlets derived from both shoot tips and first nodes. Quantitatively the effect was not a major one but similar transitions on a large scale would severely limit the usefulness of any clonal propagation scheme. Experiment 2a was therefore repeated with the 3 culture passages extended to 4 weeks each (Exp.2b). If the extent of flowering was indeed related to the length of time in culture, then more extensive transition to flowering would have been expected in Exp.2b than in Exp.2a, since the total duration of the former was 12 weeks, while that of the latter was only 9 weeks.

The data for propagule doubling times for Exp.2b are shown in Table 13. The transition to flowering has affected all explants of cvs. Telegraph and Pepinex cultured on the NAA medium (Figs 40 & 42). Cultures of cv Rebella have remained substantially in the vegetative growth phase throughout the culture period (Fig 41). Transition to flowering was extensive on NAA media; by the third passage, it has reached 100% in all cultures of cv. Telegraph and in node derived ones of cv. Pepinex. Cultures on BA were less subject to the flowering transition but by the third passage, even on this hormone, node derived cultures of cv. Telegraph were increasingly affected.

Regulation of flowering by exogenous auxins in vitro is not well documented; but it is practised in vivo on a large scale in at least one instance, that of pineapple (Clark & Kern, 1942; Wareing & Phillps,1981; Hartmann, et al, 1988). The pineapple crop is sprayed with auxins (such as 2,4-D and NAA) to induce flowering. The analogy must not be pressed too far, however, because the premature transition to flowering observed here was more genotype dependent, rather than hormone specific.

The propagule doubling times shown in Exp.2b (Table 13) are slightly longer than those in Exp.2a (Table 12); this probably has less to do with flowering than with the longer passage duration, leading to nutrient limited growth.

The previous experiments have shown that, under certain conditions, transition to flowering can interfere with a clonal propagation procedure. These observations have also been confirmed by experiments run concurrently on the longer storage of cultures(chapter 4), in the course of which it has become apparent that the temperature is one of the factors affecting the transition to flowering. It was therefore decided to investigate the effect of temperature on the clonal propagation rate and on the vegetative-reproductive phase transition in the next experiment(Exps.3a and 3b). Even though the hormone used was BA and not NAA a limited percentage of the cultures flowered at 20 and 25°C, but no flowering was observed at 30°C (Exp.3a)(Plate, 11). The propagation rate was highest at 30°C (Fig 45) for all 3 cultivars, and the length of shoots was also highest at this temperature. The most convenient temperature for clonal propagation of cucumber is therefore 30°C. In the next experiment (Exp.3b) which run over 90 days, 15 and 30 day passage-lengths have been compared. As expected, and as shown by Plate 12, the plantlets are taller after a 30 day passage than after 15 day one; but the propagule doubling times are shorter for 15 day passages than for 30 day Ones.

Callus formation was more pronounced during 30 day passages (Plate 13) than during 15 day ones. It is therefore best to keep the duration of culture passages low to 15 days in the clonal procedure.

Plate 11. (left) Effect of different temperature on growth and transition to reproductive stage in various cultivars. Note flowering at 20 and 25°C in tested cultivars. x 0.5

Plate 12. (right) Characteristic feature of plantlets regenerated in different passage duration at 30°C and after 90 days culture 0n 0.35 µM BA. x 0.5

TEMP
Cult
20°C
25°c
30°C
17d(25°c)+ 15d(20°C)
32d
17d(25°c)+ 15d(30°C)
Telegraph
Rebella
Pepinex 69

Cult
6 passages of 15 days each
3 passages of 30 days each
Telegraph
Rebella
Pepinex 69

Plate 13. Callus formation on the explants during 30 day passages at 30°C, after 3 months, cv. Pepinex. x 0.5

CHAPTER: FOUR

LOW LABOUR-INPUT MAINTENANCE OF CULTURES; PROBLEMS OF PREMATURE FLORAL INITIATION.

Although serious attempts to develop in vitro storage procedures for plants began about 16 years ago (Witther, 1988), we are far from having procedures applicable to all species.

To date there is only one report about seed storage in liquid nitrogen in cucumber (Styles et al, 1982). The viability of the seeds in normal storage condition i.e. 5°C was reduced to 79% in 600 days, but that of the liquid nitrogen in the same period was 87%. Since there was no significant differences between those treatments, we found it necessary to establish a vegetative culture storage of plants by in vitro techniques.

To establish a protocol for the low labour-input maintenance of shoot tip cultures without premature floral initiation in culture, the following two experiments were carried out:

Experiment 1. Choice of temperature.

Experiment 2. Effect of osmoticum on low labour-input maintenance and floral initiation.

RESULTS.

Exp.1 Choice of temperature.

Cultures can be stored in a state of slow growth by various means, including culture at a reduced temperature. The use of low temperature (2-10°C) for long term low growth of cultured plant cells and organs has been applied successfully to grape (Morel,1975), strawberry (Mullin and Schlegel, 1976), potato (Westcott et al, 1977) and apple (Ludergan & Janick, 1979). Due to lack of reports in cucumber low labour-input maintenance in the literature, our objective in the present experiment was to investigate the suitable temperature for the maintenance of cultures using shoot tips.

Three cultivars (as explained in chap 3) were employed in our experiment. Explants were taken from 13 days old in vitro grown seedlings and cultured in a 175 ml glass jars containing 0.35 μM BA.

After placing the explants in the specified media, they were put in an incubator with 25±1°C and with 16 hr photoperiod in 11.5 Wm^{-2} light intensity for one week to remove any probable contaminated explants . At the end of one week all the jars were shifted to 20°C and kept there for one week. At the end of the period the specified cultures were left there and the remaining were transferred to 15°C. The procedure was continued weekly from 15°C to 5°C, till the specified cultures were distributed to the allocated conditions. The cultures maintained in above condition for three months without any renewal of the media.

There were 10 replicates per treatment (1 t0 3 explants in each jar).

At the termination of the experiment the plants were shifted to normal condition i.e. 25°C and were assessed in relation to survival percentage and transition to flowering. The results are shown in Tables 18-19 and Fig 51.

At 5°C (cold room) all cultures (100%) suffered of mortality (Table 18). The plantlets in one month turned to greenish-yellow and followed by turning to yellow and then died without any further growth.

At 10°C, Although the plantlets died due to cold, they initially turned yellow with black leaf-tips (Table 18).

At 15°C, the growth response of the tested cultivars were identical. The plantlets grew very well and filled the jars. The growth of shoots and roots were Profuse. the shoots began to grow and the number of flowers increased when they were shifted to standard conditions. In relation to plant appearance, it can be added that the foliage was vigorous, but the lower leaves (old ones) were yellowish-brown at the end of three months (Plate 14). The media was also exhausted in this treatment. The first flowers were observed after 35 days after the cultures were shifted to the specified temperature. Cvs Telegraph and Pepinex produced more flowers than cv. Rebella at the beginning , but with the passage of time this cultivar also changed to flowering (Table 19).

At 20°C the general growth of the plantlets was a little better than that of cultures at 15°C (Plate 15). The other difference was that of flowering time. In this treatment the flowers appeared 5 days earlier than that of 15°C. Most of the flowers were produced on

cvs. Telegraph and Pepinex (Table 19). In all cultivars the flowers were produced in only two uppermost nodes.

Plate 14 (Top). The appearance of plantlets after 3 months maintenance on the media containing 0.35 µM BA at 15°C. Note flowering on both cultivars.

Plate 15 (Bottom). The appearance of plantlets after maintenance on the media containing 0.35 µM BA at 20°C . Note the flowering on this condition.


4/15
35 BA Tele
5/9/88
32/15


25/20
35 BA Repi
5/9/88
3/4

As regards the flower number in the above conditions, the highest numbers were observed in 20°C. Cv Rebella produced the lowest number of flowers in comparison with the others (Fig 51, Table 19).

Fig 51. Effect of different temperature on flowering after 3 months maintenance on 0.35 μM BA.

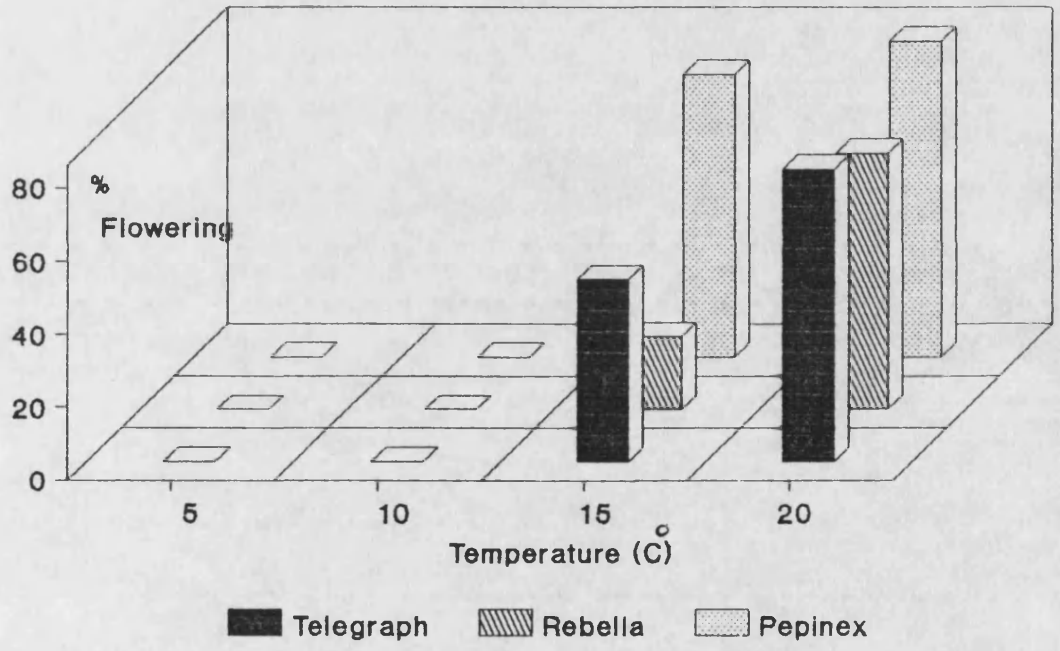


Table 18. Percentage of survivals after 3 months storage at different temperature. n= 10

Temp (C°) / Cult	5	10	15	20
Telegraph	0	0	100	100
Rebella	0	0	100	100
Pepinex	0	0	100	100

Table 19. Percentage of plants flowering after 3 months storage at different temperature. n=10

Temp (C°) Cult	5	10	15	20
Telegraph	0	0	50	80
Rebella	0	0	20	70
Pepinex	0	0	77	86
Mean	0	0	49	79

Exp 2. Effect of osmoticum on low labour-input maintenance and floral initiation.

In order to establish a suitable laboratory procedure for the low labour-input long term maintenance of cucumber tissue cultures by manipulating the culture temperature and osmoticum concentration, two temperatures (20 and 30°C) and 3 mannitol concentrations (0, 3 and 6%) were compared in the following two experiments:

a. At 20°C.

Shoot tips of 13 day old in vitro grown seedlings were used as explants. The basal medium was MS hormone free which was supplemented with 0, 3 and 6% mannitol. The cultures were kept at 20°C with 11.3 Wm^{-2} light intensity and 16 hr photoperiod for 3 month .

b. At 30°C.

All cultures and maintenance techniques were the same as that of 20°C, except the light intensity which was 12.3 Wm^{-2} .

7-10 replicates (1 to 3 explants in each jar) were used per treatment and maintained in 16 hr photoperiod at the above temperatures. Experiments were carried out with the previous three cultivars (see chapter 3) for 3 months. At the end of the period the shoot length was measured and root extension was scored from 0 to 5 (0 being the lowest root extension and 5 the highest). Survival of cultures were also assessed by the ability of plantlets to produce new growth then the plant was transferred to fresh media containing 0.35 µM BA which was used for the propagation.

After assessment of all the cultures, two of the plantlets were transferred onto a 2:1 (w/v) mixture of compost and perlite, the remaining of the plantlets were shifted to the specified storage conditions i.e. 20°C to be assessed at the end of six months. The cultures in 30°C were not suitable for extending the culture, therefore these cultures were only shifted to fresh media and compost.

There were very significant differences (p=0.01) between the treatments in shoot growth and root extension (Table 20-22).

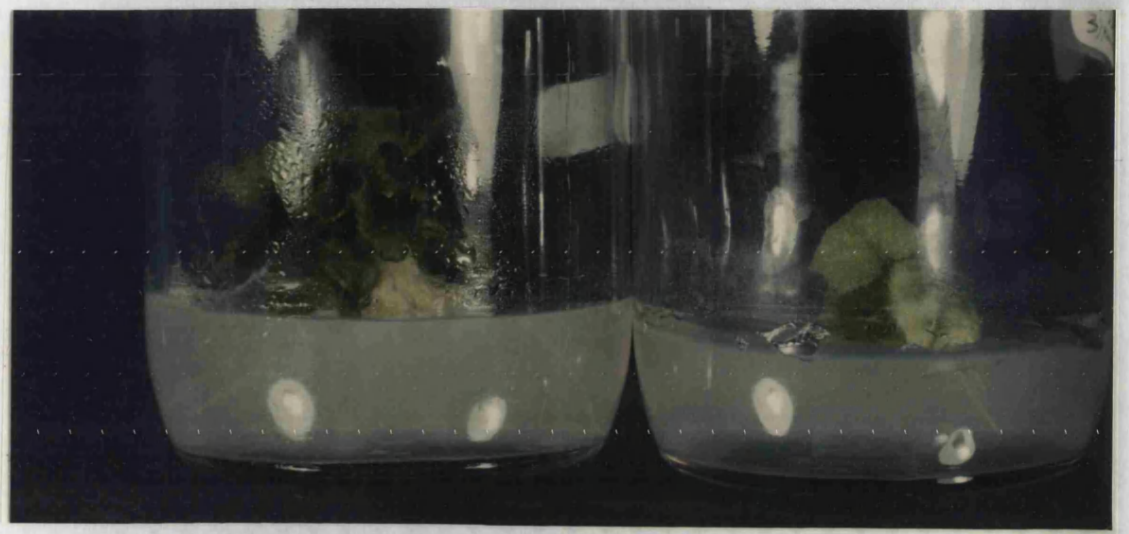
All cultivars on hormone free medium (lack of mannitol) went to flowering and produced fruits at 20°C. The plants were healthy and vigorous; there was also enough media in the jars for further culture.

Presence of mannitol in the culture medium had a retarding effect on the growth and development in all of the cultures. The plantlets in 3% mannitol were short at the end of three months (Plate 16). Only 1 out of 10 plantlets in cv. Telegraph produced flowers in this condition. When two of the plants were transferred from this treatment to the propagation media and kept in normal propagation condition (30°C) they started to grow in two weeks time and produced enough roots. When these plants were transplanted to the compost they produced multiple shoots (2 t0 3 shoots per plants). There was no difference between the weaned plants and parent plants in appearance.

All explants cultured on 6% mannitol at 20°C became brown and died during the first month of culture .

Plate 16 (Top). The appearance of plantlets after 3 months maintenace at 20°C on the media containing 3% mannitol then transferring to the media containing 0.35 µM BA, cvs. Telegraph (left) and Pepinex (right).

Plate 17 (Bottom). Effect of 3% (right) and 6% mannitol(left) on plantlets survival in cv. Rebella after 3 months at 30°C. Note the root initiation and multiple shoot at 6% mannitol.




%6 mannitol
3% mannitol

At 30°C all control plants grew very fast and the medium was exhausted before the end of the first culture period and most of the plantlets were dead, only a few of them were tip green.

The cultures on media containing 3% mannitol produced more roots but their growth was retarded. At the end of three months the leaves were brown, but the stems were swollen. The stem and axillary buds were green and alive (Plate 18).

On the 6% mannitol the leaves were brown, but the stems were shorter than that of 3%; the plantlets were more vigorous than those of 3% mannitol. The root extension was lower than 3%. There was no flower initiation in any of the cultures even in the control treatment(Plate 18).

When the plantlets were transferred to the propagation media only plantlets obtained from 6% mannitol survived very well and established better and these plants also produced multiple shoots (3-5 shoots per plantlet), one of the shoots were fasciated in this condition (Plate 17).

In general the plantlets survived and established better in 6% mannitol than those of 3% in 30°C (Plate 19).

Plate 18 (Top). The appearance of plantlets on different mannitol concentrations (control, 3% and 6% from left to right) after 3 months maintenance at 30°C. Note the leaf and stem growth.

Plate 19 (Bottom). Appearance of plantlets after 3 months maintenace at 30°C then transferring to a media containing 0.35 μM BA. Note the multiple shoots.


Rebella


6% Reb
30°C

Table 20. Effect of different mannitol concentrations on culture growth after 3 months storage at 20°C and under a 16 hr photoperiod in cv. Telegraph.

Plant growth Mannitol Concs. %	Number of original explants	Mean shoot length (cm) ± sem	Mean root extension ± sem
0	10	4 ± 0.3	3 ± 0.3
3	8	2 ± 0.4	2 ± 0.7
6	10	dead	dead

Table 21. Effect of different mannitol concentrations on culture growth after 3 months storage at 20°C and under a 16 hr photoperiod in cv. Rebella.

Plant growth Mannitol conc. %	Number of original explant	Mean shoot length (cm) ± sem	Mean root extension ± sem
0	12	4 ± 0.3	4 ± 0.6
3	7	1 ± 0.0	0.0 ± 0.0
6	9	dead	dead

Table 22. Effect of different mannitol concentrations in culture growth after 3 months storage at 20°C and under a 16 hr photoperiod in cv. Pepinex.

Plant growth Mannitol Conc. %	Number of original explant	Mean shoot length (cm) ± sem	Mean root extension ± sem
0	15	5 ± 0.8	3 ± 0.5
3	13	1 ± 0.0	1 ± 0.3
6	10	dead	dead

Discussion

The importance of conservation of genetic resources, can not be over emphasized (Frankel and Hawkes, 1975; Henshaw, 1979; Ford-Lloyed and Jackson, 1986). Germplasm storage in the form of seeds is easy, but for the species whose seeds have poor viability, or which do not produce seeds such as seedless watermelon, the plants must be maintained in the vegetative state under field conditions. The maintenance of living material by conventional methods is expensive, laborious and risky (Arora & Bhojwani, 1989).

Tissue culture offers an alternative method for conserving germplasm in a vegetative state so that a large number of plants can be produced when required.

Micropropagation is an essential part of in vitro conservation of germplasm. In chapter 3 we explained the possible procedure of cucumber propagation system. In this chapter we employed shoot tips as an explant for establishing a low labour-input maintenance protocol, since shoot tips have been identified as a suitable material for long term preservation germplasm by low temperature storage in different species (Lundergan and Janick, 1979; Miedema, 1982). Unfortunately till now such a system has not been exploited in cucurbits in general and in cucumber in particular. Therefore in two major experiments the possibility of establishing a low labour-input maintenace protocol was explored.

When a range of temperatures i.e. 5°C to 20°C were studied the results showed that the temperature 10°C and the lower temperature were not suitable regimes for maintenance of cultures in our conditions. The results are not in contrast with normal conditions of the plant in nature. Since the plant is a warm-season one and grows best in temperatures range of 18-30°C (Yamagushi, 1983); the plants suffer chilling at temperature below 10°C.

The response of cultures in the temperatures 15°C and 20°C was transition to reproductive phase, the only difference between those treatments was that, the number of flower buds on the plants maintained in 15°C were lower than that of 20°C. It also seemed that they were suffering from low temperatures (Plate 14, Table 1). As is shown in table 19 the response of the cultivars to this treatment was also different.

From the results it became clear to us that the only way to establish a long term storage protocol was suppressing the plants growth by exploiting retardant chemicals in one of 15 or 20°C regimes. The results of Chatterjee and Lama (1977) and Staritsky et al (1986) support this propose.

They demonstrated that the culture storage depends on the origin of the plant and its need of temperature range for suitable growth in the nature. Therefore in relation to the value of the mannitol in slowing the growth of cultures in some plants (Mathias, 1980; Westcott, 1981; Henshaw, 1982) the second experiment was designed. In this experiment the basal medium was MS hormone free medium, since the possible influence of any hormone in plant growth was avoided. This medium was used as a control. The other treatments were the MS medium supplemented with 3 and 6% mannitol. All the cultures were kept in an incubator with 20°C and 16 hr photoperiod for three months without adding any nutrient or transferring to fresh media. Similar experiment was carried out in the same time and kept at 30°C.

The presence of mannitol in the culture medium had a retarding effect on the growth and phase change of the plants in all cultivars (Plates 16-19, Tables 20-22). The response of cultivars to mannitol was a little different, for instance 3% mannitol inhibited floral initiation 100% in cvs. Rebella and Pepinex, but it was not very affective in cv. Telegraph; 10% of

the plantlets in this treatment went flowering. In comparison all the plantlets in control initiated flowers and produced fruits.

Although 3% mannitol appears to be a useful treatment in all cultivars in 20°C, the use of 6% mannitol had no advantage since all of the cultivars were dead in this treatment . At 30°C the response of the cultures were very different than those of 20°C. There was no flower initiation in any of the cultures. In general the cultures on 6% mannitol looked vigorous than those of 3% . In all mannitol treatments the stem of the cultures was thick and green but the leaves were yellow to brown (Plate 18).

The cultures maintained on 6% mannitol in 30°C were healthier than that of 3% and survival percentage (60%) was higher than that of (10%) in 3%. When these treatments are compared with that of the 3% mannitol (90%) in 20°C, there is no doubt that in our conditions the 3% mannitol in 20°C is the best low labour-input maintenance protocol for cucumber.

The important points in our results were that : a) the cultures that survived produced multiple shoots in both 3% mannitol in 20°C and in 6% in 30°C (Plates 16,17 and 19) this was in agreement with results of Henshaw (1982). b) survival of weaning plants is another case which can be used to grow the plants in the field or glasshouse or to provide material for another cultures. and c) that the mannitol concentration could be determined in respect to genotype and storage temperature.

CHAPTER : FIVE

Effect of hormones and environmental factors on sex expression of cucumber *in vitro*.

Regulation of sex is very important for fruit vegetable production. In some species, such as cucumber this has been possible by manipulating environmental and chemical factors. Mainly the effects of environmental factors such as photoperiod and temperature have been reported (Nitsch et al, 1952; Fukushima et al, 1968; Matsuo & Fukushima, 1969; Lower et al, 1975; Rute & Butenko, 1978; Takahashi & Saito, 1986). Studies on various plant growth regulators revealed that exogenous application of auxins such as IAA and NAA, ethylene, ethrel and growth retardants enhance the femaleness, whereas GA_3, silver nitrate ($AgNo_3$) have an opposite effect (Peterson & Anhder, 1960; Mitchell & Wittwer, 1962; El-Ghamriny et al, 1988).

All the results mentioned above were obtained using intact plants. In vitro culture technique was found to provide an ideal tool for the investigation of plant growth regulators on the sex modification without interference from leaves or other organs (Galun et al, 1963; Dickens & Staden, 1988). Flower bud and immature inflorescence cultures offer unique opportunities for this type of study.

Cucumber flower buds were first cultured by Galun et al (1962). They cultured young floral buds still at bisexual stage. They succeeded in modifying flowers of a genetically male strain (York State pickling) to develop into female ones.

The culture of immature inflorescences rather than individual buds has the following advantages:

a) each inflorescene carries a large number of buds.

b) ease of obtaining the explants.

b) ease of obtaining the explants.

c) no reports of immature inflorescene cultures exit for cucumber, although such explants have been cultured in other species.

In this chapter, three experiments were carried out with view to examine the effect of some environmental inputs (in particular, growth regulators and photoperiod) on sex expression in selected genotypes of cucumber in culture. Auxins and /or ethylene have been reported to promote the expression of the female phenotype both in vivo and in vitro (Peterson & Anhder, 1960; Mitchell & Wittwer, 1962; El-Ghamriny et al, 1988); experiments 1A, 1B and Exp.2 were designed to investigate this aspect of sex expression on immature inflorescence cultures.

Experiment 3 was concerned with the effect of photoperiod on the sex expression in shoot tip derived- plantlets cultured in vitro. The smaller the explants, the smaller the the likelihood that observed changes in sex expression are caused by signals arising outside the reproductive structures being investigated. Immature inflorescences as used in Exps. 1A, 1B and 2 are a good compromise in this respect between individual buds and whole plantlets. In experiments using whole plantlets as in Exp.3, interpretation has to take into account that the exogenous stimulus, in this case photoperiod elicit the primary response in an organ other than the reproductive structure.

The genotypes used in these experiments are listed in Table 22.a: The experiments were as follows:

Table 22.a. Genotypes used in experiments to study sex expression.

Genotypes	Normal phenotype *in vivo*	used in Exp. No
F_1 (Telegraph x Chipper)	Predominantly androecious	1A, 1B & 2
F_1 (Perfection x Ottawa)	Predominantly androecious	1A, 1B & 2
Perfection (P_1)	Predominantly androecious	1A, 1B & 2
Ottawa (P_2)	predominantly hermaphroditic	1A, 1B & 2
Telegraph	Predominantly monoecious, sometimes gynoecious	1B & 3
Rebella	Gynoecious	3
Pepinex	Gynoecious	3

The normal phenotype for each genotype referred to in Table 22.a is the one exhibited by greenhouse grown plants under a 12 hour photoperiod in the temperature range 25°C-30°C. The genotypes were chosen partly to represent a range of normal phenotypes from androecious to gynoecious and partly because flowering in vitro was relatively straightforward to induce in them.

Other culture conditions, such as temperature and media comparisons were also varied from one experiment to the next , which excludes the possibility of making comparisons between the results of individual experiments, but this does not affect the interpretation of results within each experiment, which is consistent and meaningful.

The experiments were as follows:

Exp.1. Effect of IAA on sex expression in immature inflorescences.

A. with 16 hour photoperiod, at constant 25°C.

B. with 8 hour photoperiod, at 23°C/17°C day and night temperature.

Exp.2. Effect of 2-chloroethylphosphonic acid (ethrel) on sex expression in immature inflorescences, with 16 hour photoperiod at constant 25°C.

Exp.3. Effect of 8, 12 and 16 hour photoperiods on sex expression in hormone free medium at constant 20°C in shoot tip-derived explants.

RESULTS.

Exp 1. Effect of IAA on sex expression in immature inflorescences.

A. with 16 hour photoperiod, at constant 25°C.

One of our problems in carrying out this experiment was the limitation of seed supply. The seeds were donated by a private donor. We received on average 7 seeds for each genotype. The problem with these genotypes was that the seedlings developed into the reproductive phase and made the multiplication difficult. To avoid the problem the plantlets were propagated at 30°C then shifted to 25°C under a 16 hr photoperiod with light intensity of 2.9 Wm^{-2}. The shoots were maintained for 23 days in the above condition to induce flower initiation.

When the first floral buds which were too small to determine sexes visually were initiated, the five uppermost nodes were excised and used as explants, consisting of a single infloresence initial bud maximum size 0.7 mm , on a stem piece maximum length 5mm.

The immature inflorescence was excised and cultured in 9-cm sterile plastic Petri dishes.

The culture method used in our experiment was essentially the same as that of Galun et al (1963). The basal medium was the White's basal medium which was used by Galun.

The stock solution and the amount used for the basal medium are listed in Table 23, Appendix 2.

For preparing the medium, stock solutions I through to VII and coconut milk (CM) were passed through 0.2 μm Acrodisc Sterile non-pyrogenic filter and was added to double distilled water to make one-fourth of total volume of the medium. Agar and sucrose were dissolved in distilled water to make up the remaining three-fourth volume of the medium and were autoclaved.

When the autoclaved part was cooled to about 50°C it was mixed with the filtered part then the IAA in the specified concentrations i.e. 0, 1 μM (0.2 mg/l) and 2 μM (0.4 mg/l) were filtered into the basal medium to make the culture medium. Afterwards the medium was dispensed into the specified plastic Petri dishes.

3 to 6 explants were placed on the media of each Petri dish, sealed and then placed in the growth room with the same condition given above.

The explants were cultured for three months. At the end of the culture period, the explants were assessed by counting the male and female buds produced in each treatment. The effect of different hormones was also evaluated in relation to leaf and root initiation on the cultures.

The results are shown in Figs 51-54 and Table 24, Appendix 2.

On the basal hormone free medium the leaves were pale green and their growth was not satisfactory. Root induction was genotype-dependent. As seen in Plates 20 and 21 root induction occurred on genotypes P_2 (Ottawa)and F_1 (Perfection X Ottawa), but not in other genotypes (Plate 22).

Fig 51a. Effect of IAA on sex expression of immature inflorescences after 90 d culture at 25°C and under 16 hr photop.

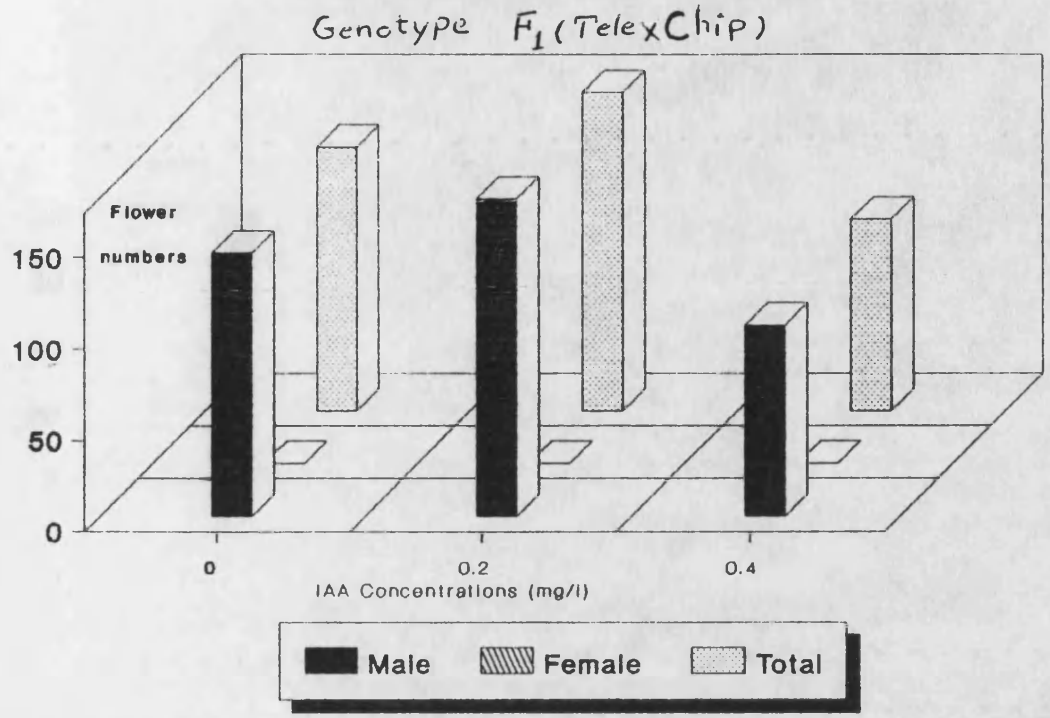


Fig 52. Effect of IAA on sex expression of immature inflorescences after 90 d culture at 25°C and under 16 hr photop.

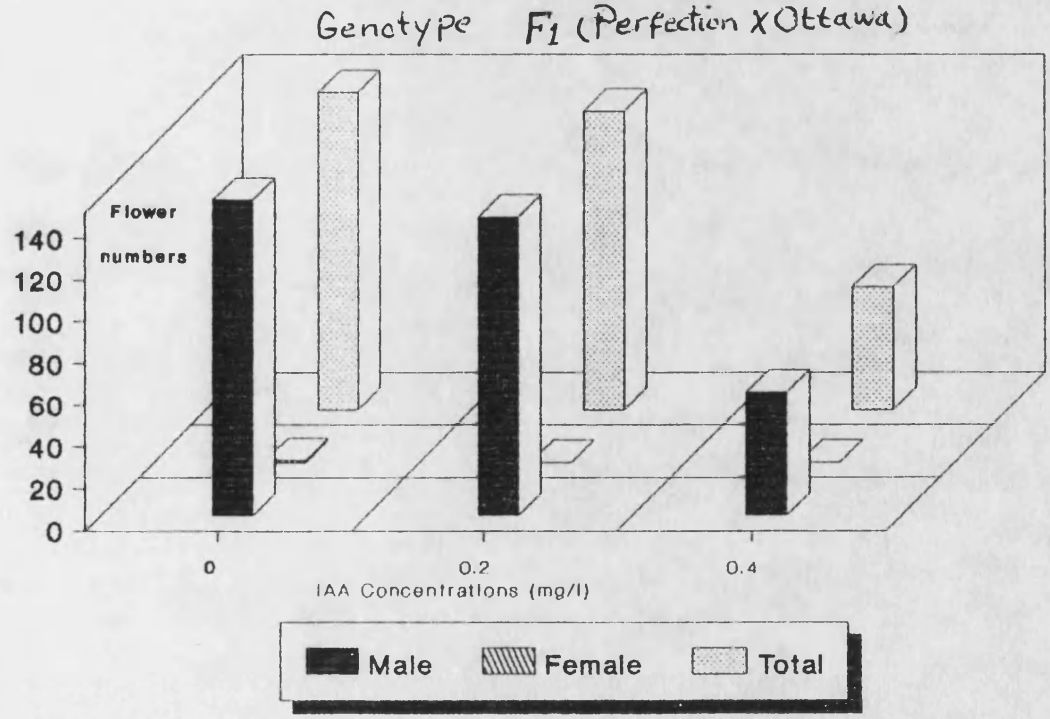


Fig 53. Effect of IAA on sex expression of immature inflorescences after 90 d culture at 25°C and under 16 hr photop.

Genotype P_1 (Perfection)

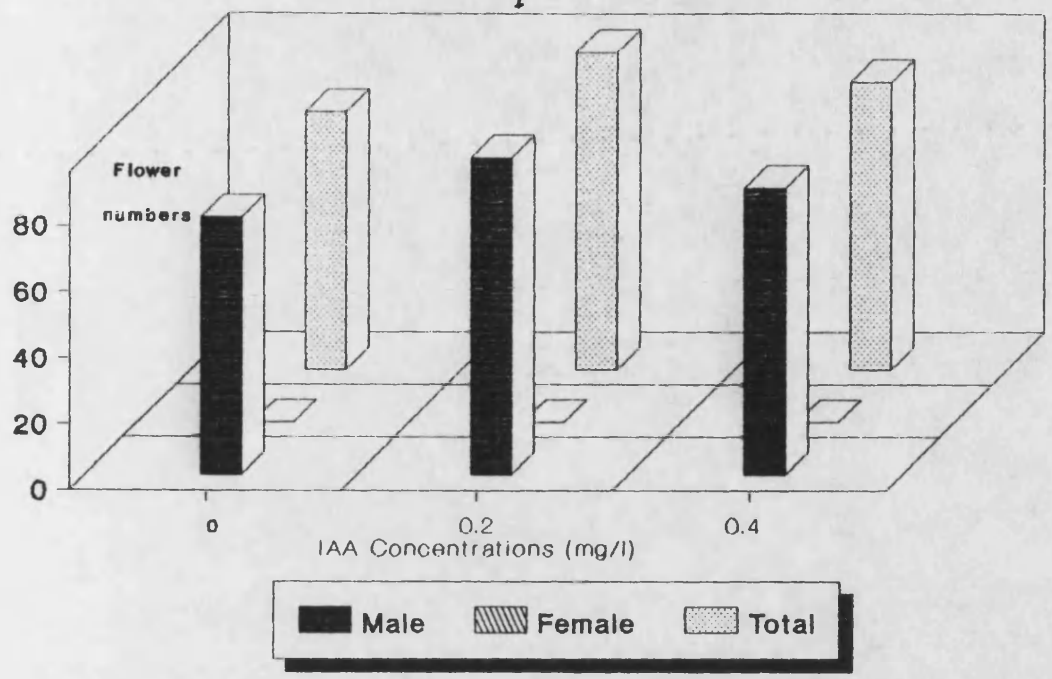


Fig 54. Effect of IAA on sex expression of immature inflorescences after 90 d culture at 25°C and under 16 hr photop.

Genotype P_2 (Ottawa)

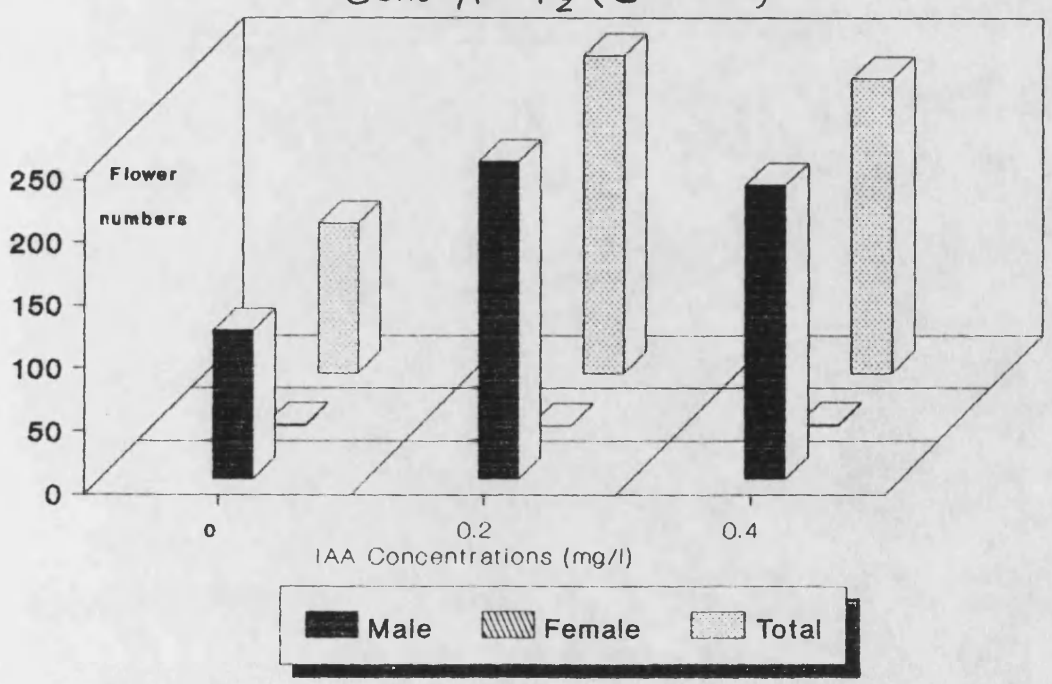


Plate. 20 (left). Sex expression and explant growth on hormone free medium containing CM, after 3 months, in genotype P_2 (Ottawa). Note the root initiation and fruit set. x 3.2

Plate . 21 (right). Sex expression and explant growth on hormone free medium containing CM, in genotype F_1 (Perfection X Ottawa) . Note the pale green leaves. x 3.3


Pg=nbnb
14131817
→5151817
IAA=0

Although the flower bud's growth was not very satisfactory, it seemed the hormone free medium was useful for floral bud initiation. Only two fruits were produced in the whole treatment and those were on genotypes F_1 (Perfection X Ottawa) and P_2 (Ottawa)(Plates 20 and 23). There was no callus or root initiation in these explants.

The medium containing 1 µM (0.2 mg) IAA had a positive effect on the flower initiation. 12 to 15 floral buds were obtained from some of the genotypes on this treatment. Leaf production was very low on this medium. Root initiation occurred in these explants. No sex modification occurred on the media consisting of 1 µM (0.2 mg) in any of the genotypes tested. The flower number and size of the individual floral buds were increased in this media in comparison with those of the hormone free ones. The other difference was in the number of developed and opened flowers, which it was more pronounced on this medium (Plate 24), it was more genotype-dependent than hormone concentration.

On the media containing 2 µM (0.4 mg) IAA, the number of leaves was decreased in comparison with that of 1 µM (0.2 mg) IAA. The root extension was genotype-dependent, for instance root extension was observed in genotype P_1 (Perfection), but not on genotype P_2 (Ottawa)(Plates 25 and 26). The largest floral buds were obtained on this media. Flowers opened on this treatment earlier than on the other ones (Plate 26). There was only one fruit set in all the genotypes on the media and it was on genotype P_2 (Ottawa). No root initiation was observed on this explant.

Plate 22 (Top). Sex expression and growth of immature inflorescence , genotype P_1 (Perfection) on hormone free medium containing CM. Note lack of root initiation. x 3.3

Plate 23 (Bottom). Fruit formation on hormone free medium containing CM in genotype F_1 (Perfection X Ottawa), after 3 months culture. x 3.2

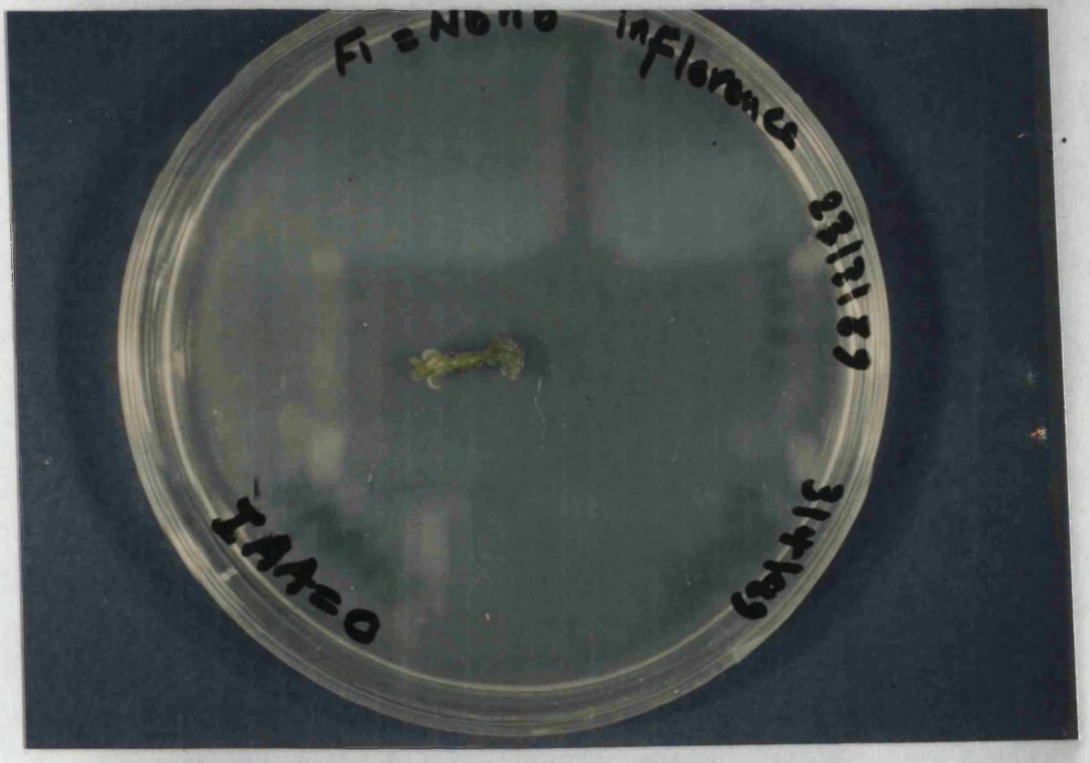

Florence
TAMCO

Plate 24 (Top). The effect of basal medium containing CM and 1.0 μM (0.2 mg/l) IAA on floral bud initiation, genotype P_2 (Ottawa). x 1.2

Plate 25 (Bottom). Effect of media containing 2.0 μM (0.4mg/l) IAA on floral bud and root initiation of explants, genotype P_1 (Perfection). x 1.2


P2=nbnb nonbit
19/8/89
IAA=1.0


P1=NbNb bitter
19/3/89
IAA=2.0 MM

Exp 1. Effect of IAA on sex expression in immature inflorescences.

B. with 8 hour photoperiod, at 23°C/17°C day and night temperature.

Plant material was the previous three genotypes used in the earlier experiment.

The basal medium now consisted of MS hormone free medium plus White's CM free basal medium. IAA in the range of 0, 0.1, 0.3 and 1.0 mg/l was added to the medium in the same way as explained in previous experiment. Cultures after excision were planted on 9-cm plastic Petri dishes and transferred to the growth room with 23°C day and 17°C night temperature and illuminated for 8 hr photoperiod at an intensity of 2.9 Wm^{-2}.

The explants were cultured for 3 months in the above conditions. At the end of culture period, the male and female floral buds were counted.

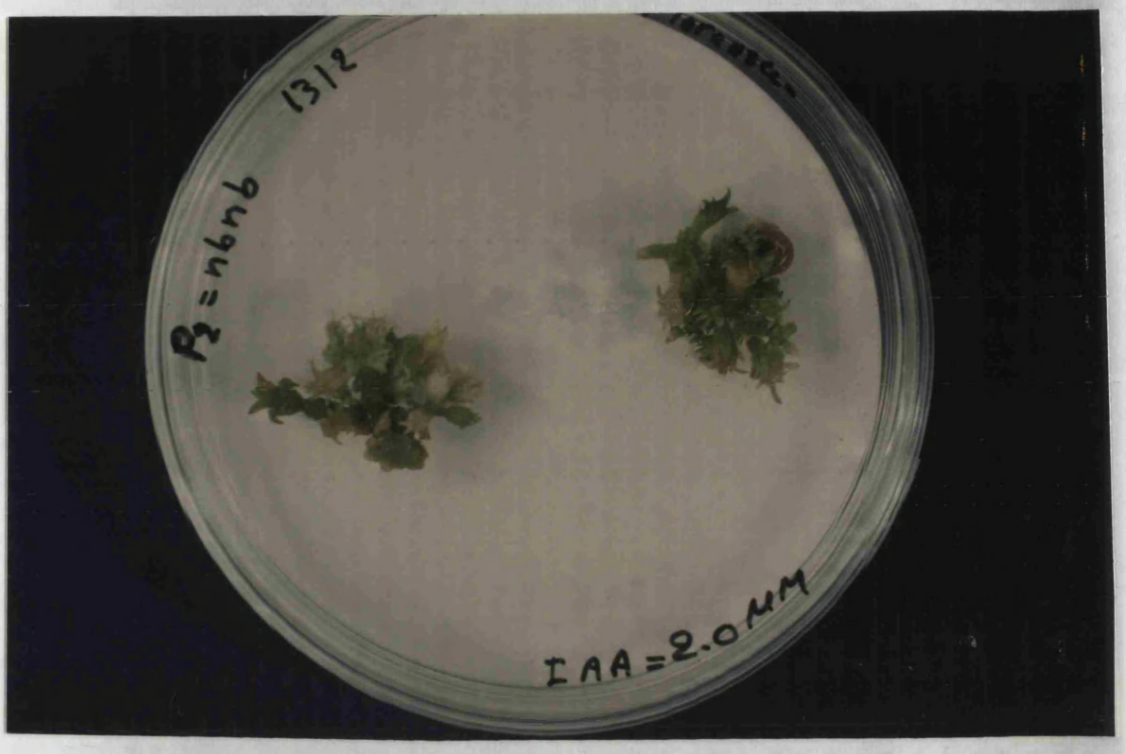
The results are shown in (Table 25, Appendix 2 and Plates 27-32).

Floral buds in the basal medium (control) were small in comparison with in vivo condition (Plate 27). The buds produced in vitro resembled those of in vivo and each had a normal complement of floral organs i.e. sepals, petals, stamens. Each flower also had a short thick pedicel (Plates 27-28). An adventitious shoot was formed on the excised inflorescences. Normal leaves, thick roots were initiated on these shoots. As shown in (Table 25, Appendix 2) the lowest number of floral buds were produced on this medium. Sex

modification only occurred in genotype P_2 (Ottawa) on this medium on which the lowest female flowers were produced (Table 25, Appendix 2).

Plate 26 (Top). Effect of media containing 2 μM (0.04 mg/l) IAA on sex expression , genotype P_2 (Ottawa). Note lack of root initiation and fruit formation. x 1.2

Plate 27 (Bottom). Effect of hormone free medium without CM on floral bud initiation and explant growth. x 1.2


R2=nbnb 1312
IAA=2.0MM


P2=nbnb inflorenc
node-1-6
7104
111S169
IAA=0

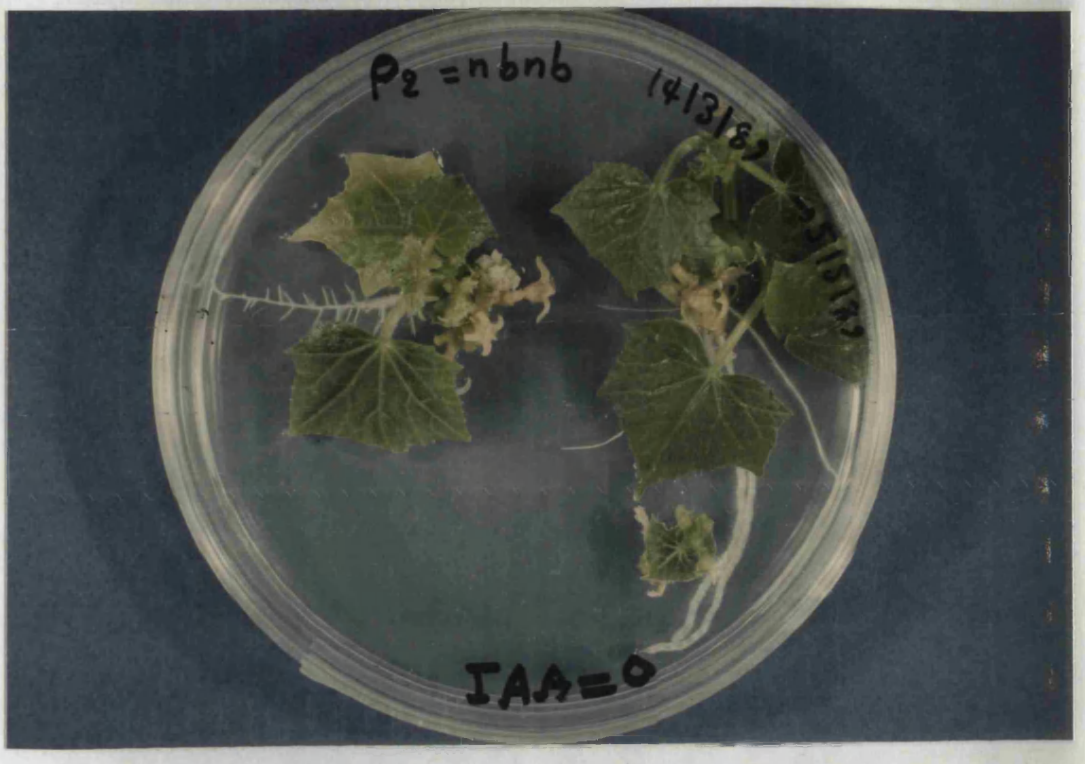
The buds grown in the medium containing 0.1 mg IAA could not produce more leaves. Their number decreased in comparison with control. The number of floral buds increased in this treatment. Floral buds produced on this medium were smaller than those of hormone free medium. Root extension was also decreased on the media. Roots were thinner than those obtained from control (Plate 29). Media consisting of 0.1 mg IAA had no effect on sex expression of the tested genotypes (Table 25, Appendix 2).

In the basal medium supplemented with 0.3 mg IAA the number of the flowers was higher than in the other treatments in all genotypes (Table 25, Appendix 2). Flower size was smaller, mostly closed. There was no female flower on this treatment in any of the genotypes tested (Table 25, Appendix 2). Root initiation was obtained in most of the explants. Roots were thicker than those obtained on 0.1 mg IAA (Plate 30). A few leaves were also produced on this treatment.

The explants grew on the medium containing 1.0 mg IAA, possessed a well developed floral organs very similar to those produced in the flowers in vivo, but in miniature size (Plates 31-32). There were more opened flowers on this media than those of the other tested treatments. The individual flowers had long pedicels. Very long and thin roots were also obtained on this media. The number of leaves produced on this treatment was the lowest among the tested media. Although the flowers produced on the media containing 1.0 mg IAA were lower than that of 0.3 mg IAA, both genotypes P_1 (Perfection)and F_1 (Perfection x Ottawa) produced one fruit on this treatment (Plates 31-32).

Plate 28 (Top). Root initiation and shoot regeneration on hormone free medium without CM in genotype P_2 (Ottawa). x 1.2

Plate 29 (Bottom). Sex expression and root initiation on the media containing 0.1 mg/l IAA, genotype P_2 (Ottawa). x 1.2


P2 = nbnb
14131
IAA=0


P2 = nbnb
1312
14131
IAA=0.1

Plate 30 (Top). Effect of media containing 0.3 mg/l IAA on root and floral bud initiation, genotype F_1 (Perfection X Ottawa).

Plate 31 (Bottom). Fruit formation on the media containing 1.0 mg/l IAA, genotype P_1 (Perfection). Note lack of root initiation. x 3.4


F1 = Nbnb inflore 9/6/89
[AA] = 0.3 mg/l

Exp 2. Effect of 2-chloroethylphosphonic acid (ethrel)on sex expression in immature inflorescences,with 16 hour photoperiod at constant 25°C.

For investigating the effect of ethrel on sex expression of immature inflorescences, two genotypes i.e. F_1 (Perfection X Ottawa) and P_2 (Ottawa) which were used in our previous experiments were also employed in the present experiment.

The explants were obtained by clonal multiplication in 30°C under a 16 hr photoperiod in an aseptic condition. Multiplied shoots were maintained for 25 days in growth room (explained in Exp 1 and 2 of the present chapter) to initiate the floral buds. When the first floral buds were formed they were cultured on the basal medium (the same composition as the earlier experiment) supplemented with different ethrel concentrations. Three concentrations were used in this experiment: 0, 10^{-2} and 10^{-1} mg/l.

After placing the explants on the specified media the Petri dishes were sealed and transferred to the growth room. The growth room condition was the same as previous experiments.

The explants were cultured in the above conditions for two months. At the end of the culture period , number of male and female flowers produced in various treatments was counted and used for sex modification assessment. The leaves and shoots obtained in some of the cultures were also recorded.

The results are shown in (Figs 55-56 and in Table 26).

In hormone free medium, the root and leaf initiation was low. Root initiation was genotype-dependent (Plates 30). Leaves did not grow on this media; all the leaves were

necrotic. As shown in (Table 26), the hormone free medium had the least effect on sex modification. In average 11 flowers were produced in each explant.

On the medium supplemented with 10^{-2} mg ethrel, the number of total floral buds formed on each explant decreased in comparison to that of hormone free (Table 26). In spite of the lower floral bud initiation on this media, the highest number of fruits was obtained on it (Fig 56, Table 26). Leaves formed on the media were necrotic; the lowest root extension was observed .

The medium containing 10^{-1} mg ethrel had no positive effect on floral bud initiation, since there was no significant difference between this media and that of control (Table 26). As shown (Plate 33) two fruits were produced in one explant, which was a rare accident during our study in sex expression. The highest sex expression was also obtained in this media (Table 26). Root and leaf initiation was not high, but the symptoms of necrosis were not as much as in the two other treatments. The Number of floral buds initiated on each explant was decreased in comparison to that of the other two media (Table 26)

Fig 55. The effect of ethrel on sex expression of immature inflorescences in cv. F1(Perfection x Ottawa)

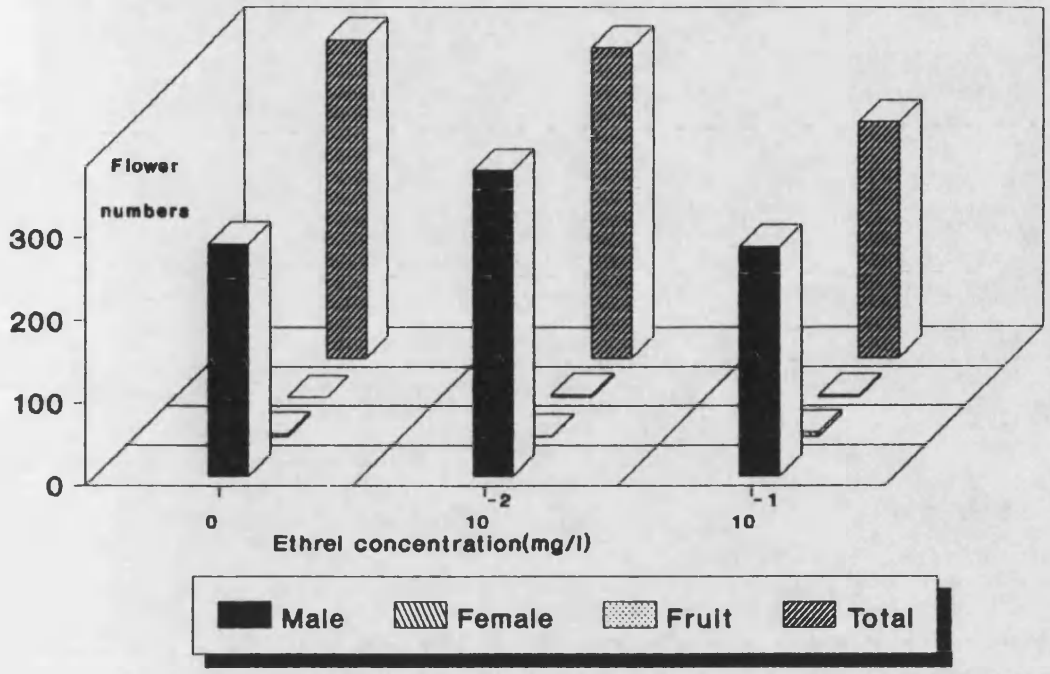


Fig 56. The effect of ethrel on sex expression of immature inflorescences in cv. F1(Perfection x Ottawa)

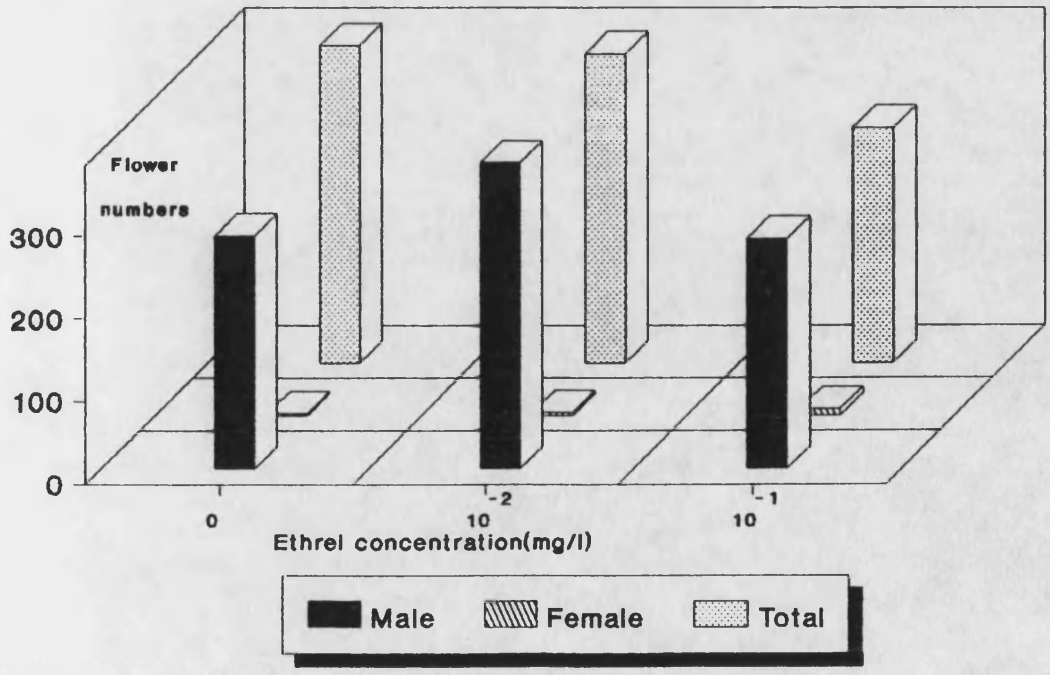
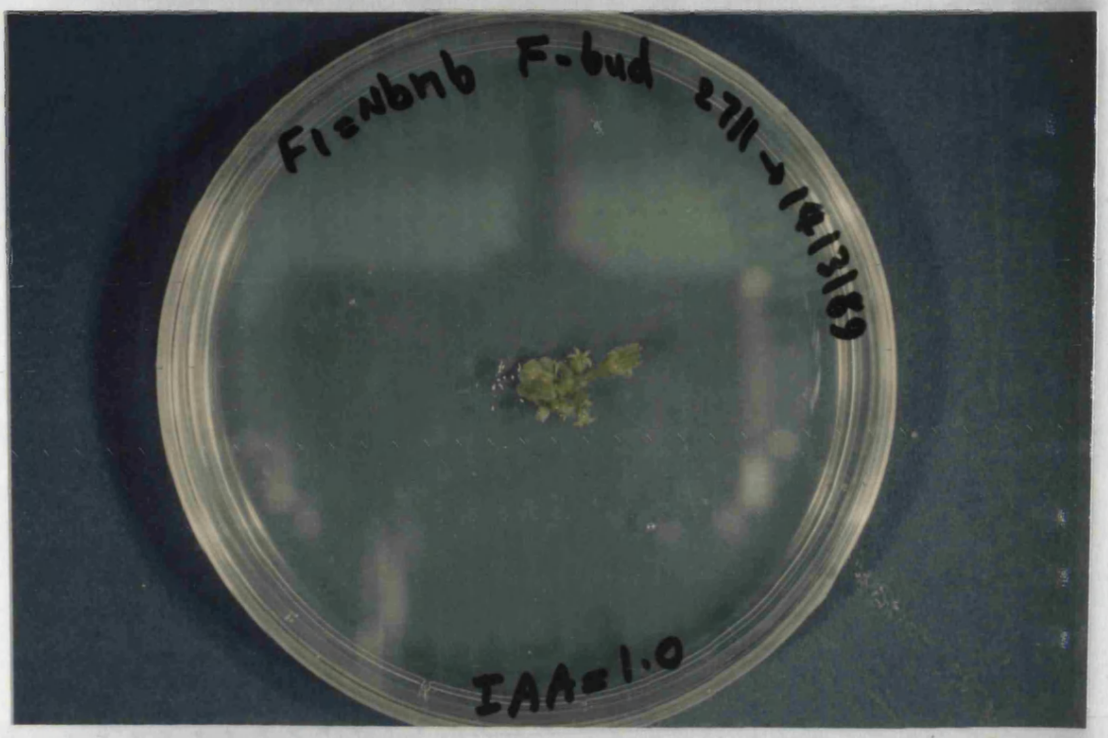


Plate 32 (Top). Fruit formation on the medium containing 1.0 mg/l IAA, genotype F_1 (Perfection X Ottawa). x 1.2

Plate 33 (Bottom). Effect of ethrel concentrations on sex expression in immature inflorescences. x 1.4


F1=Nbnb F-bud 2-11 → 14/3/89
IAA=1.0


Hormone-Free
10 -1
10 -2

Table 26. Effect of different ethrel concentrations on sex expression in immature inflorescence. Maximum flower bud size in each explant 0.7 mm.

Sex express / Horm conc/Cult		No of original explants	No of flowers obtained			Flower/ explant
			male	female	total	
F_1 (Perfec X Ottawa).	0	36	382	3	385	11
	10^{-2}	45	371	4	375	8.3
	10^{-1}	53	279	8	287	5.4
P_2 (Ottawa)	10^{-1}	11	51	0.0	51	4.6

Exp 3. Effect 8,12 and 16 hour photoperiod on sex expression in hormone free medium at constant 20°C in shoot tip-derived plantlets.

Three cultivars i.e. Telegraph, Rebella and Pepinex were used in this experiment. Shoot tips were obtained from 13 day old in vitro grown seedlings. The explants cultured on MS hormone free medium .

The cultures were maintained at 20°C under three different photoperiods i.e. 8, 12 and 16 hr for 58 days. The light intensity was 9.3± 0.5 Wm^{-2}. 7 to 11 replicates were allocated for each treatment (1 to 3 explants per 175 ml jar).

At the end of the culture period, the number of male and female flowers was counted. The fresh weight of the plantlets was recorded; shoot length was measured and the number of nodes produced on each plantlets was also counted.

The results are shown in Fig 61 and Tables 27 and 29, Appendix 2. The rest of the results are presented in Figs 56-59 and Tables 27 and 29, Appendix 2.

Photoperiod was found to be highly affective in modulating sex expression in the gynoecious genotypes Rebella and Pepinex. The gynoecious phenotypes was stable in both cultivars under a 12 hour photoperiod, and in Rebella , also under 16 hrs, Pepinex was monoecious, but predominantly male under a 16 hour photoperiod, while both Rebella and Pepinex were affectively androecious under 8 hours daylight . On the other hand, the normally androecious cultivar Telegraph remained predominantly androecious under all 3 photoperiods, although a smmall percentage (10%) of flowers were female in 12 hour photoperiod. The total number of flowers induced in all cultivars was also photoperiod-dependent (Fig 61.a, Table, 27). The highest number of flowers was obtained under short days, while long days were less efficient in inducing flowers.

The earliest flowers produced in 27 days after the beginning of the culture in 12 hr. The latest flowers appeared in 36 days and it occurred in long days.

Fig 57. Effect of photoperiod on fresh weight on MS hormone free medium at 20°C.

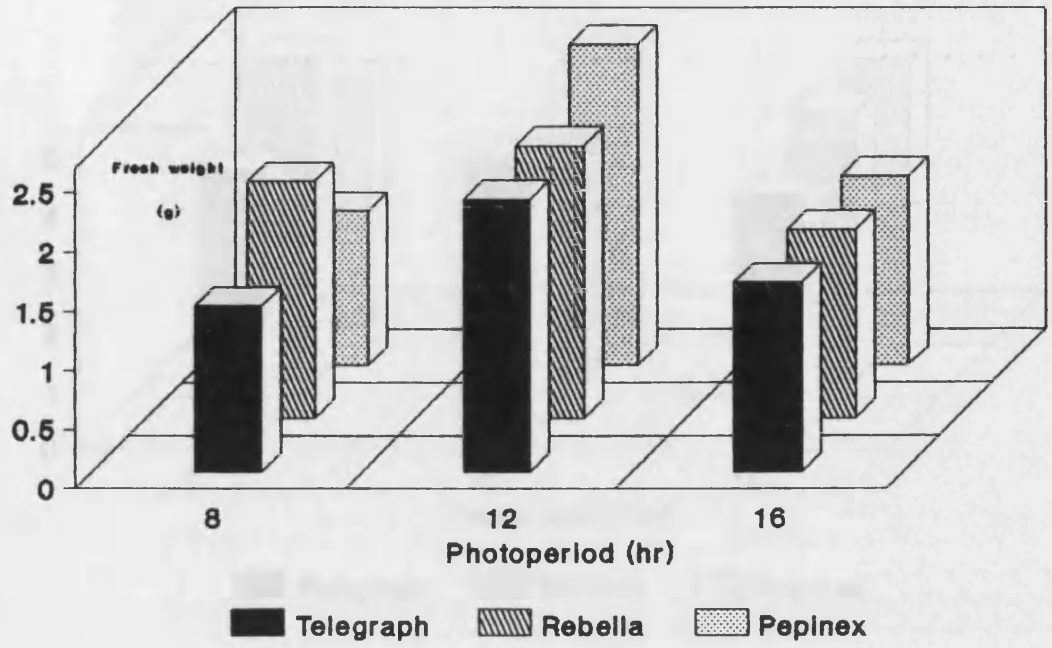


Fig 58. Effect of photoperiod on shoot length on MS hormone free medium at 20°C.

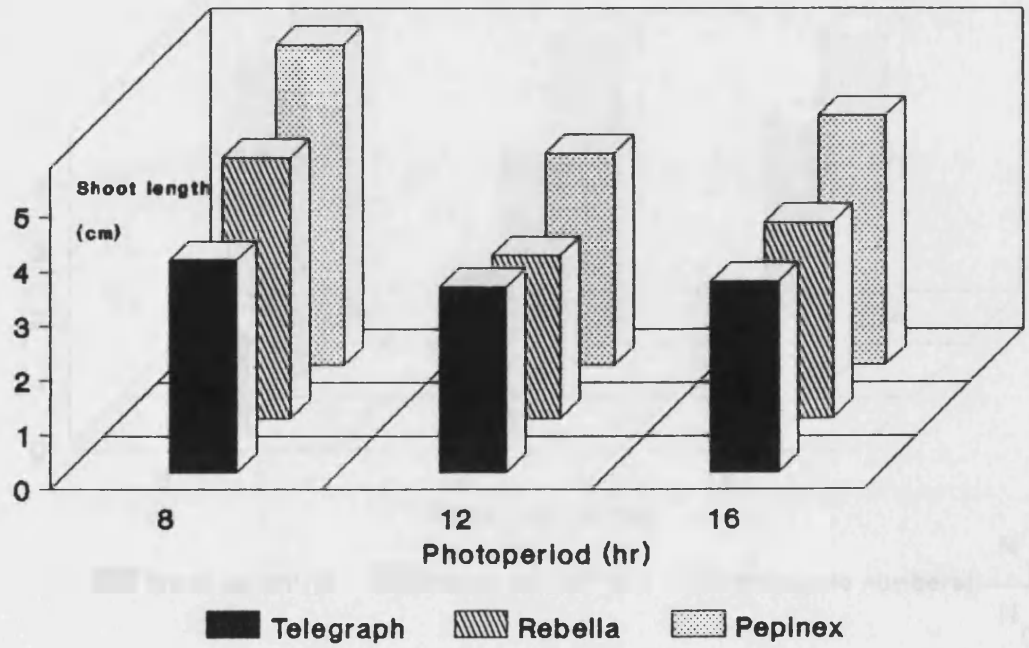


Fig 59. Effect of photoperiod on proliferation rate on MS hormone free medium at 20°C.

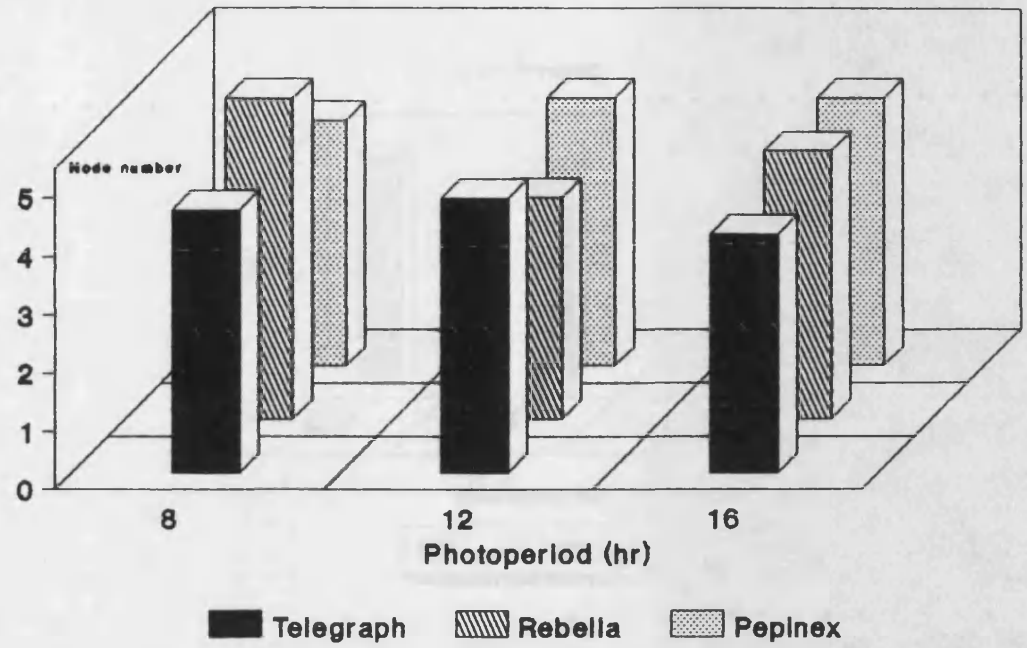


Fig 60. Effect of photoperiod on plant growth and propagule proliferation on MS hormone free medium at 20°C.

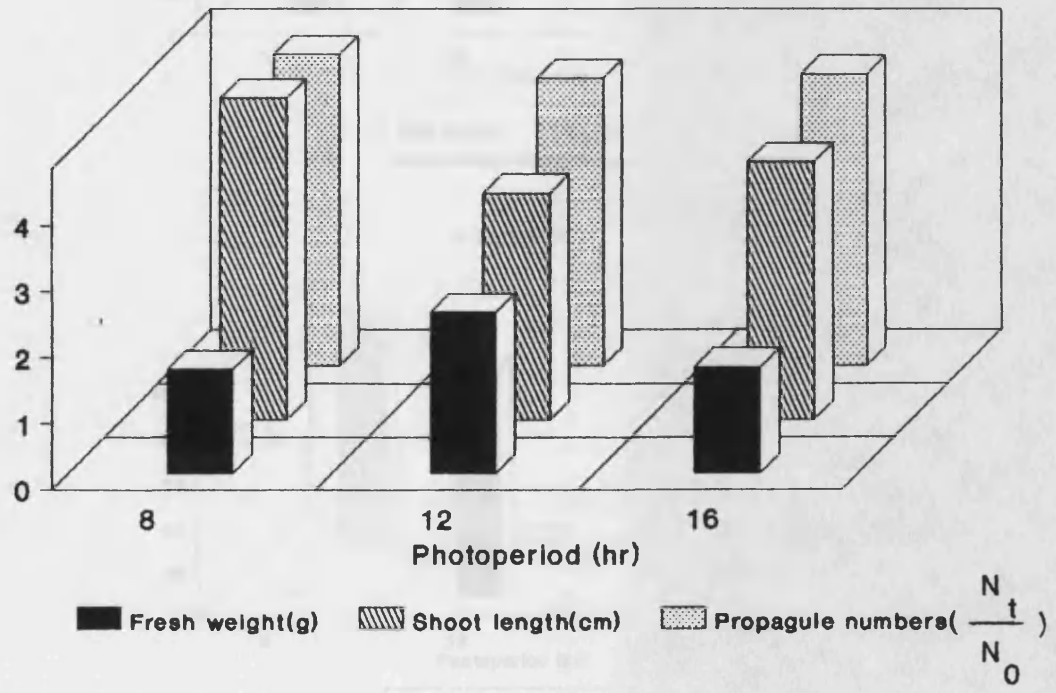
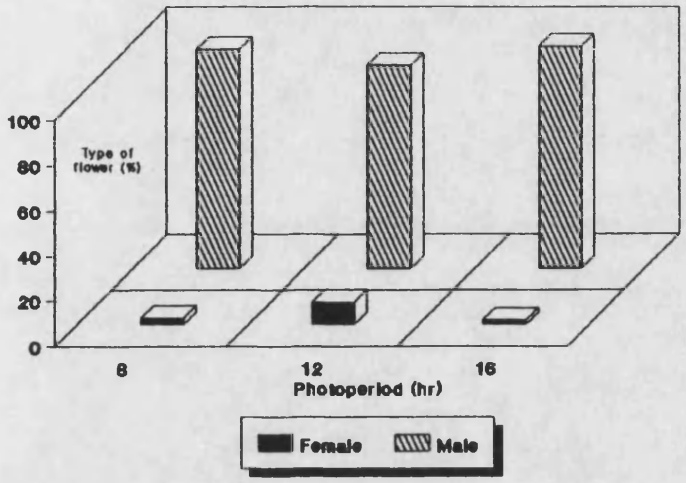
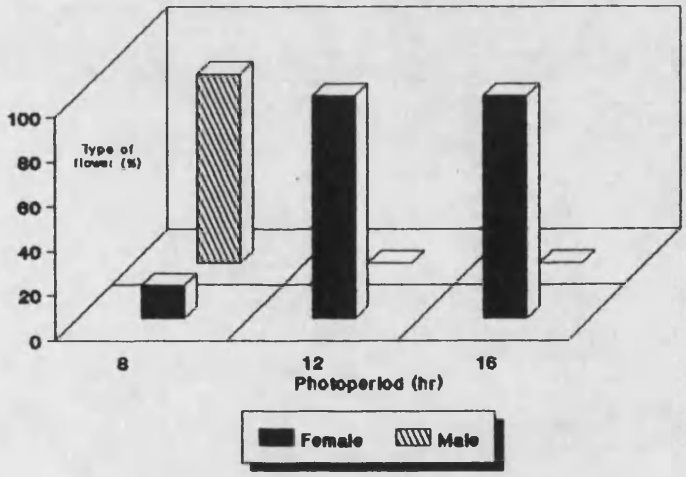


Fig 61. Effect of photoperiod on sex expression in shoot tip-derived plants on hormone free medium in vitro.

a. cv. Telegraph



b. cv. Rebelle



c. cv.Pepinex

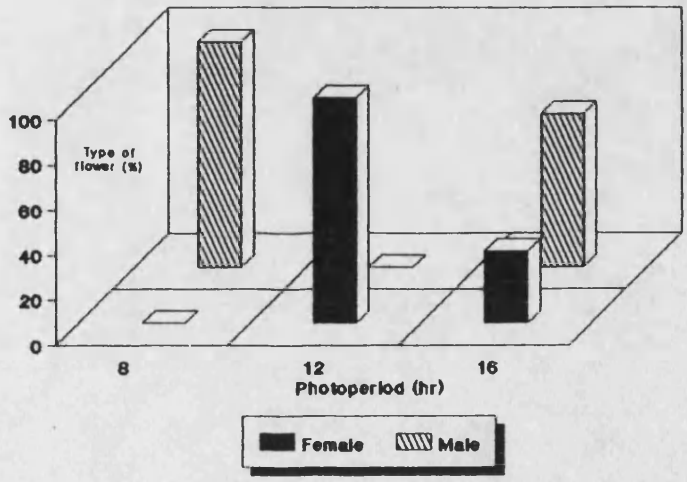


Fig 61.a Effect of different photoperiod on sex expression on MS hormone free medium at 20°C for all cultivars.

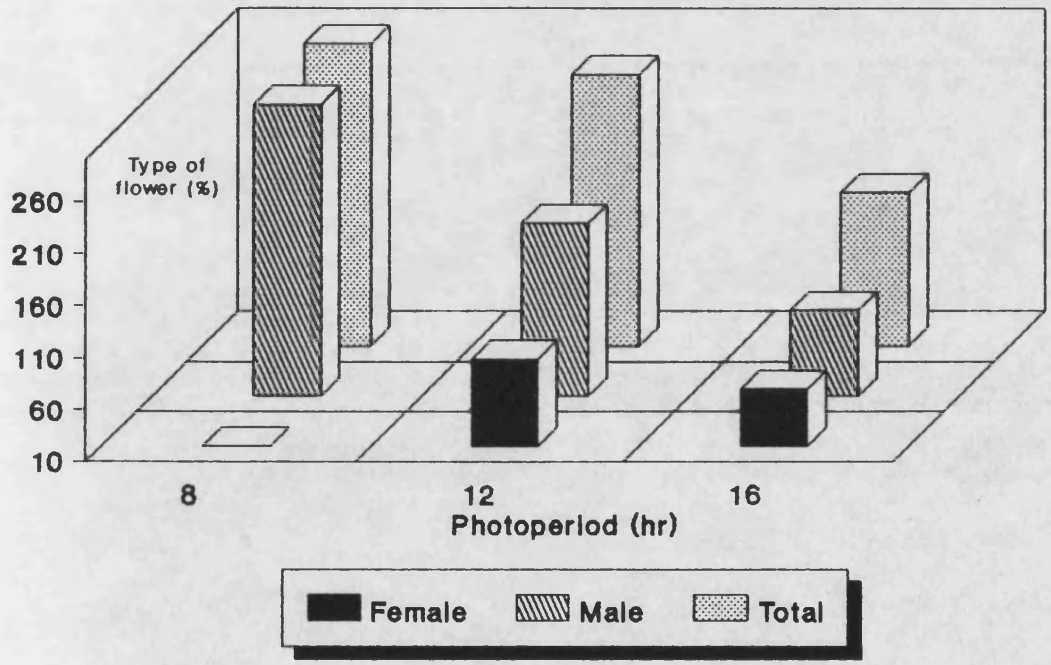


Plate 34. Male flower production at 8 hr photoperiod and 20°C, cv. Pepinex in vitro.

Fresh weight of the plantlets was also affected by the different photoperiods. The greatest fresh weight was obtained in 12 hr and the lowest one in short day condition (Figs 57 and 60, Tables 28 and 30, Appendix 2).

Although photoperiods tested in these conditions had no significant difference on shoot growth, the tallest shoots were obtained in 8 hr and the shortest in 16 hr (Figs 58 and 60, Tables 28 and 30, Appendix 2).

The treated plantlets with different day length did not show any significant differences in proliferation rate. The highest number of nodes were obtained in short day length and the lowest one in 12 hr (Figs 59-60, Tables 28 and 30, Appendix 2).

Discussion

In experiment 1A and 1B, five genotype were utilized. Three androecious, one hermaphrodite and monoecious. IAA has been applied to immature inflorescences in culture in increasing concentrations. The application of auxin has left the normal male sex expression essentially unchanged. It is often stated that auxins promote female sex expression in cucumber and other cucurbits (Heslop-Harrison, 1957; Matsubara, 1977). One example of this is shown in Table 26.a below; the results show the effect of auxin application to young plants in vivo

Table 26.a: The effect of auxins on sex expression in Cucumis sativus.
Data of Laibach & Kribben, 1950, from Heslop-Harrison, 1957.

Auxin	No. of female flowers in successive leaf axis	Average no. of female flowers in first seven axils (10 plants).
	1 2 3 4 5 6 7	
0.1% -naphtalenacetic acid	4 - 8 1 10 11 8	45.5
0.1% -indoleacetic acid	- - 1 2 1 2 2	11.1
0.1% 2,4-dichloro phenoxyacetic acid	- - - - 2 - -	2.9
Control	- - 1 2 - - 2	2.2

Galun et al (1963) investigated the effect of IAA application at 0.3 ppm to excised floral buds (0.3 to 0.7 mm long) in vitro (cv. York State Pickling) was to increase the percentage of female flowers from 89% to 100%. The authors refer to these buds as potentially male. In view of the high percentage of female flowers among the hormone free this statement seems dubious, unless excision and culturing in vitro per se were comparable with those of Galun in that auxin application has had only a very small effect on sex expression in immature inflorescences in vitro, Table 24, Appendix 2.

Rudich et al (1969) suggested that the observed effects of auxin on sex expression in vivo result from auxin-induced ethylene formation. Yang (1969) in his experiments with cucumber in vivo found that ethrel released ethylene in the presence of plant tissues. Other investigators found that ethrel was remarkably effective in increasing femaleness in cucumber in vivo (Mc Murray & Miller, 1968; Sims & Gledhill, 1969). The effect of various plant growth regulators, including ethrel on sex expression was investigated by Matsubara, 1977 in cucumber in vitro. He found that ethrel as the most effective substance for the promotion of femaleness.

In Exp.2, in the present work, for the investigation of the effects of ethrel on sex expression in immature inflorescences in vitro only 3 female flowers were obtained out of a total of 385 floral buds. Similarly, Matsubara (1977) reported the development of one female flower out of a total of 57 on hormone free medium. The cultivar was Sagami-Hanjiro.

The main effect of the application of ethrel in concentrations of 10^{-2} and 10^{-1} ppm in the present experiment was to reduce the total number of flowers and the number of flowers per explant (Table 26). The feminizing effect of ethrel was small: in the F_1 hybrid Perfection x Ottawa 1% of flower buds became female on 10^{-2} ppm ethrel application, and less than 3% on 10^{-1} ppm ethrel. The all-male sex expression in cv. Ottawa remained unchanged.

In Matsubara's experiments, the feminizing effect of ethrel application was more pronounced: at 10^{-2} ppm ethrel, 23% female flowers were obtained while at 10^{-1} ppm ethrel, the percentage of female flowers was 9%. The two sets of results are comparable only in a qualitative sense, because of differences in genotypes, explant-type and non-hormone medium components; but both sets of experiments indicate that ethrel did have a small effect in promoting femaleness in cucumber inflorescences in vitro.

The third experiment in this series was designed to study the effect of photoperiod on sex expression using shoot tips that were vegetative at the time of excision. The three genotypes used were the same as those used in chapter 3 for micropropagation; Telegraph is monoecious, with male flowers predominantly in the early reproductive phase, while Rebella and Pepinex are genetically gynoecious.

The main feature of this experiment was the effect of photoperiod on sex expression in the two gynoecious genotypes (Fig 61). Under 12 hour photoperiod the normally exclusively female phenotype was observed in both Rebella and Pepinex. Under a 16 hour photoperiod, however the phenotype of Pepinex showed monoecious characteristics with male flowers predominantly, while Rebella remained solidly gynoecious. The most striking change of phenotype occurred under an 8 hour photoperiod where both the normally gynoecious genotypes exhibited androecy.

On the other hand, the sex expression of Telegraph remained fixed in the normal male mode whatever the photoperiod, although a small tendency towards femaleness could be observed under a 12 hour photoperiod. It therefore seems that, at least in the genotypes tested here, manipulation of sex expression through altering the photoperiod is more effective in the female to male direction, than in the opposite sense.

On average over all cultivars were tested, increasing photoperiod from 8 hour to 16 hour had a significant (P= 0.05) effect on the total number of flowers (Fig 61.a, Tables, 27& 29, Appendix 2). The highest number of flowers were obtained in short day regime and the lowest one in long day. Therefore, these cucumber plants could be classified as short day plants, which is in agreement with the results of Rute & Butenko (1978). Thus the occurrence of some sex expression in Galun's work (1962, 1963) is indicated.

The maximum vegetative phase occurred in long day regime, which is very similar to Rute & Butenko's(1978) results.

Finally taking into account the other investigators results about sex expression in plants (Heslop-Harrison, 1972; Vince-Prue, 1975; Frankel & Galun, 1977; Sidorsky, 1978) it could be said in conclusion, that the effect of hormonal action might be variable, depending on many circumstances, such as the hormones concentration, the method and time of their application, environmental factors, the age and responsiveness of the receiver explants.

Although the present results point to the involvement of some of the hormones in sex expression of the cucumber plant, the study of sex expression in vitro is in initial stage. Therefore further work is necessary to make clear their physiological and biological action in modification and expression of sex.

CHAPTER: SIX

Stability of the bitterness trait in plants regenerated from callus of heterozygous dominant donor plants.

Cucurbitacins, terpen compounds that impart a bitter flavour to the foliage and occasionally to the fruit, are under genetic control(Robinson & Whitaker, 1962).

Bitterness of cucumber plants is controlled by one dominant gene (Phatak & Singh, 1950; Robinson & Whitaker, 1962) with the result that bitter fruit can often not be distinguished morphologically from non-bitter and are thus responsible for many cases of poisoning (Enslin et al,1954).

Andeweg (1959)found a spontaneous mutant lacking cucurbitacins and breeders have used this gene to breed non-bitter cucumber (Robinson & Whitaker, 1962). Non-bitterness can be judged simply by tasting them (Andeweg & De Bruyn, 1959).

It is evident that genetic changes occur in plant tissue culture and that these changes are transmitted to regenerated plants and their progeny (Larkin et al, 1984). Callus derived-plants as well as their progeny show some genetically determined phenotype variability-somaclonal variation (Larkin & Scowcroft, 1981). Somaclonal variation has been widely described both in cultured cells and for plants regenerated from callus (Semal, 1986). So far there has been one report available on somaclonal variation observed in cucumber (Malepszy & Nadolska-Orczyk, 1989). Variability was manifested in traits such as type of growth, male sterility, mosaic type leaves and xanth type chlorophyll change. In other species, successful selection has been achieved for specific traits such as resistance to herbicides (Chaleff & Parsons, 1978; Jones,1985; salt Nabors et al,1975) and common scab in potato (Gunn et al, 1985).

Due to lack of any information for bitterness trait in literature in vitro, it was decided to study the potential somaclonal variation for non-bitter trait.

The ability to regenerate plants from cultured cells or callus at high frequencies is important for successful application of tissue culture technology to crop improvement.

Although in vitro techniques for some of the cucumber cultivars have been developed, plant regeneration from callus has not succeeded very well (Orcyzyk & Malepszy,1985; Trulson & Shahin, 1986; Kim et al, 1988). In this chapter, not only the possibility of high frequency plant regeneration from cotyledon but unexpanded leaf explants was also investigated. When a good regeneration system was established the stability of bitterness was also tested. To achieve the above objectives the following experiments were carried out:

Exp 1. Callus induction and plant regeneration from cotyledon-derived callus.

Exp 2. Shoot regeneration from callus derived from unexpanded leaves.

Exp 3. Assessment of bitterness in regenerated plantlets.

RESULTS.

Exp 1. Callus induction and shoot regeneration from cotyledon-derived callus.

To establish a reliable protocol for plant regeneration 10 cultivars (genotypes) were used in this experiment (listed in Table 31, Appendix 2).

Based on the preliminary results on the cotyledon which direct shoots were obtained at the proximal end (data unpublished). Therefore these sections were used in the present experiment. Cotyledons for shoot regeneration was previously used in cucumber (Wehner &

Locy, 1981; Trulson & Shahin, 1986; Kim et al, 1988)without attention to the location of the responsive polarity.

Cotyledons obtained from 10 day-old in vitro grown seedlings were transversely cut off into three equal parts and the one from the proximal end was used for the explant. They were placed with their abaxial side on the surface of callus induction medium in 9-cm plastic Petri dishes.

Callus induction medium was that of Kim et al (1988), it consisted of Ms hormone free medium supplemented with sucrose (30 g/l), meso-inositol (100 mg/l), thiamin HCl (0.8 mg/l), nicotinic acid (2.0 mg/l) and pyridoxine HCl (0.8 mg/l). 0.5 µM 2,4-D and 5 µM BA. This was the best callus induction medium in Kim et al's (1988) work. Therefore it was employed in this experiment. PH of the medium was adjusted to 5.7.

Cultures were transferred to growth room at 25± 1°C with 16 hr photoperiod of 2.9 Wm^{-2}, white warm fluorescent light.

After 4 weeks the induced calli were planted on the shoot regeneration media consisting of basal medium (described above) supplemented with 0.5 µM NAA and 0.5 µM BA.

After two weeks time the calli induced the shoot tips. They were cut off and the rest of the callus transferred to the fresh media with the same composition. The process continued till the calli turned brown and died.

At the end of culture period (2 months) the regenerated shoots were counted and assessed.

The results is shown in Table 31 (Appendix 2).

Callus induction first began on the excised edges of the explants and then extended to the middle section.

The callus characteristics was varied in different genotypes from compact green to greenish white friable one. Genotype P_1 (Telegraph) could not induce callus in the same period, in which the callus induction was not occurred in comparison with the other ones. In this genotype the cotyledons only expanded and thickened.

Although the optimum callus induction was not occurred in all genotypes, it seemed satisfactory for most of them.

Transferring calli to shoot regeneration media was useful for few of them, especially for genotype Telegraph (Bookers seed Ltd) and Telegraph (Sutton Seed Ltd).

A general feature of the plantlets obtained from calli was the premature flowering.

Finally from this experiment it was found that the shoot regeneration capacity of the calli was genotype-dependent than the hormone in the culture medium. Most of the tested genotypes failed to induce shoot on the medium, therefore the calli gradually turned to brown and were dead.

Exp 2. Shoot regeneration from callus derived from unexpanded leaves.

Adventitious organogenesis and embryogenesis can induce high levels of somaclonal variation and are useful tool in breeding programmes (Evans & Sharp, 1986; Predieri & Malavasi, 1989). The abundance of leaf material in efficient micropropagation system makes it an ideal source of adventitious organogenesis or embryogenesis, especially in the case of limited supplied seed.

A few works has been reported in the area of plant regeneration from cucumber leaf derived-callus (Malepszy & Nadolska-Orczyk, 1983; Nadolska-Orczyk & Malepszy, 1984). All those regeneration was through embryogenesis. The investigators have found difficulties in obtaining high frequency of regeneration of the whole plants with normal morphogenesis.

Since one goal of this research was to develop a regeneration protocol, that may generate culture-induced variation, therefore it was decided to search a method yielding high regeneration frequencies on unexpanded leaf derived-callus.

Due to limited seed supply for genotype (Nbnb), which had the bitterness trait, it first was clonally multiplied, then the plantlets were used to provide leaf explants for the experiment. Each unexpanded leaf with 0.3-0.5 cm length was excised from the shoot tip. There was no petiole at the base of this leaf. Each leaf was placed with its underneath on the callus induction medium.

Callus induction medium was the same as that medium was used in earlier experiment. The explants were planted on 9-cm plastic Petri dishes containing 20-25 ml of callus induction medium. 5 to 10 explants cultured on each Petri dish.

Cultures were placed in an incubator adjusted at 30°C and 16 hr photoperiod provided by warm white fluorescent tubes at an intensity of 11.5 Wm^{-2}.

After about 3 weeks when the calli was induced, 0.3 g callus pieces were cut and transferred to Petri dishes containing different shoot regeneration media (listed in Table, 32).

Shoot differentiation began in the form of green islands on the callus surface after two weeks in this medium (Plate 37). Green islands were carefully cut off to pieces approximately 0.3 g and put on the fresh medium but in the specified composition. The first shoot regeneration occurred in 2 weeks on the medium.

The shoots were cut off, the remaining calli was divided into the pieces and planted on the fresh media of the same composition. The process was performed weekly in the same way till the calli lost their shoot regeneration capacity. At the end of each passage the shoots were counted and the treatments were assessed

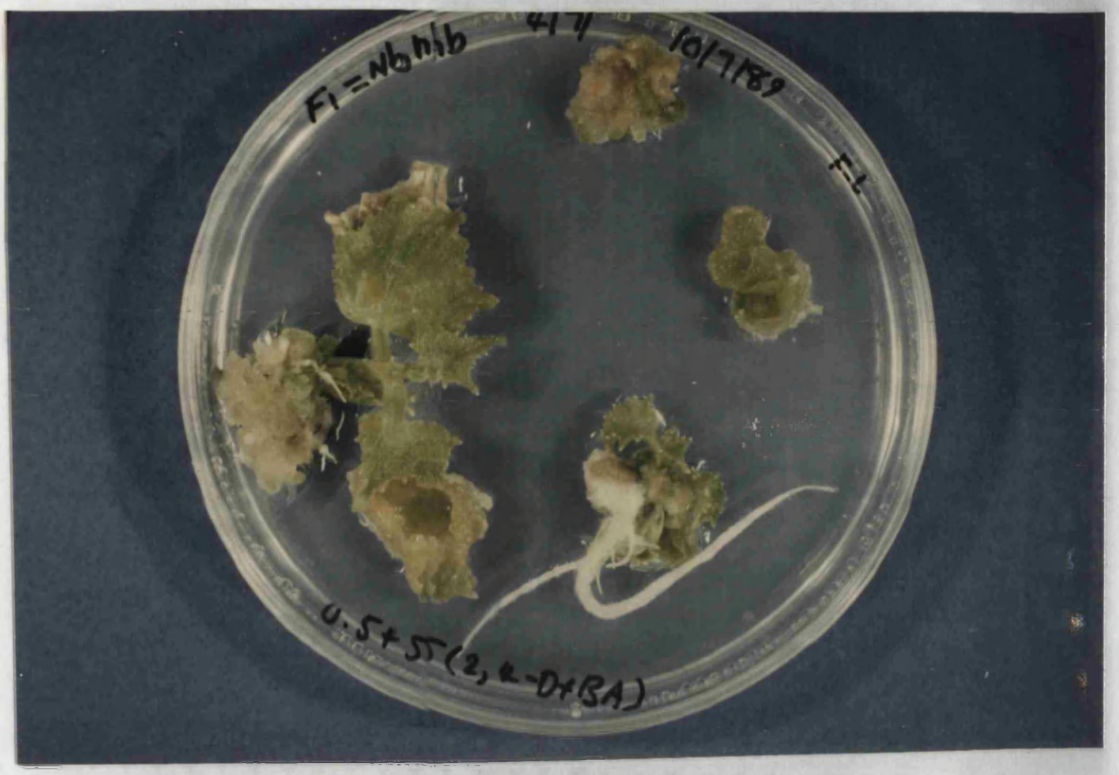
R_1 plantlets were used for shoot regeneration in the best shoot regeneration media i.e. 0.25 µM BA and the results were compared with the other treatments.

All the results are shown in Table 32. Shoots were regenerated only in two types of the media i.e. 0.25 µM BA and the callus induction one. The shoots obtained from callus induction medium were glassy and their quality was not satisfactory (Plate 35). The 4 remaining media failed to induce shoots. As seen in (Plate 36) these media varied in callus growth, by extension of the culture period they turned brown and died.

The media containing 0.25 µM BA produced the highest number of shoots (Table 32). the R_1 plantlets-derived calli produced the lowest regenerants (Table 32).

Plate 35 (Top). Shoot regeneration on callus induction media, genotype F_1 (Nbnb). Notice the quality of regenerated shoots. x 1.1

Plate 36 (Bottom). Effect of different shoot regeneration media on shoot formation of the leaf-derived callus. x 0.6


F1=Nbnb 417 10/7/89
F1
0.5+5S (2,4-D+BA)

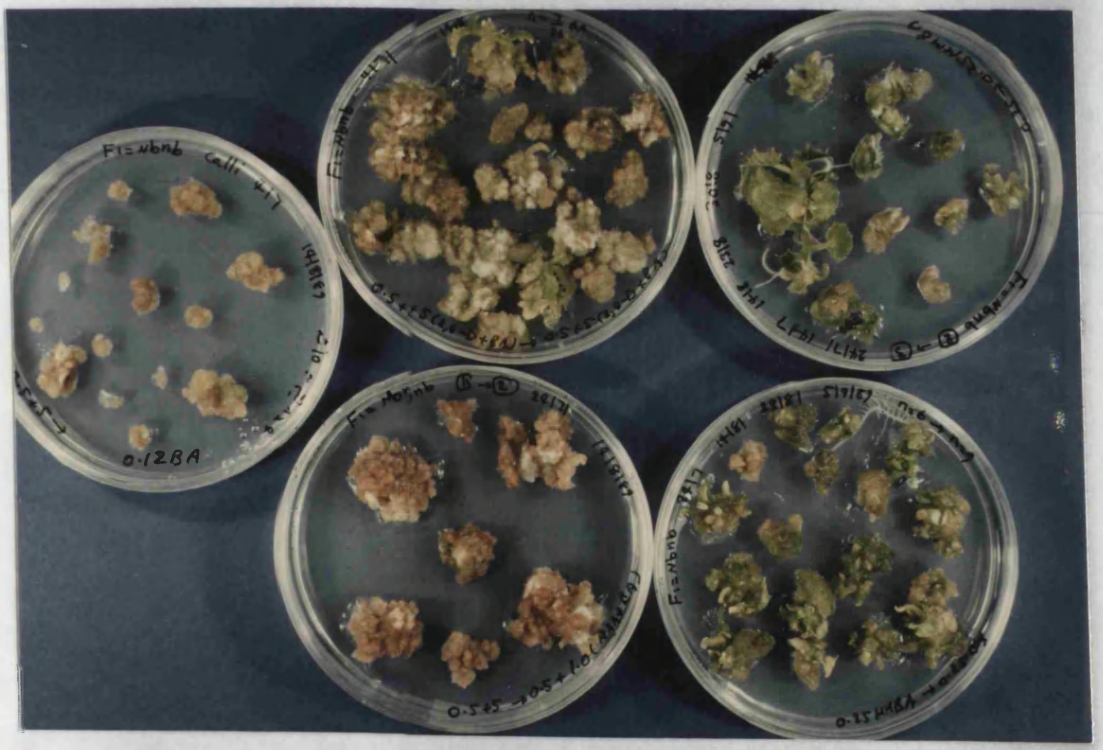

F1=Nbnb
0.12BA

Table 32. Shoot regeneration from callus derived from leaves of in vitro grown-plantlets on different media in 12 weeks of culture. Genotype F_1 (Nbnb).

Shoot regeneration		No of original explants	Total no of regenerated shoots	No of shoots per total explants
Hormone conc (µM)				
2,4-D/NAA	BA			
0.5 2,4-D	5.0	22	72	3.3
0.5 NAA	1.0	15	0.0	0.0
0.5 NAA	0.5	15	0.0	0.0
0.5 NAA	0.25	27	0.0	0.0
0.0	0.12	23	0.0	0.0
0.0	0.25	22	214	9.7
0.0	0.25*	93	115	1.2

* Explants from R_1 Plantlets regenerated from callus

Exp 3. Assessment of bitterness in regenerated plantlets.

The established regeneration system i.e. 0.25 μM BA were adopted for shoot regeneration for assessment of bitterness in R_1 generation. In addition, callus induced from plantlets regenerated from callus of R_2 generated plantlets were also tasted, 115 plantlets from 93 explants were regenerated.

747 R_1 plantlets from 194 leaf-derived calli were produced.

The 3 uppermost leaves of the R_1 regenerants were chewed until the bitter flavour was tasted. 10 minutes intervals was allocated to lapse between the samples and mouth was rinsed with drinking water. Two parental genotypes NbNb, nbnb homozygous for bitter and non-bitter respectively were tasted to be compared with genotype Nbnb. 3 uppermost leaves of those parents were chewed in the same way as explained for regenerated shoots. Genotypes NbNb and nbnb were very bitter and non-bitter respectively.

Out of 747 plant regenerated , 736 shoots were very bitter (like the parental Nbnb, and homozygous NbNb), and 11 were found to be slightly bitter, a non-bitter phenotype not found on any of the regenerants.

Among the R_2 generation, 2 plantlets had the slightly bitter phenotype and 113 the very bitter one.

Discussion.

The analysis of some main factors such as explant source, explant type and regeneration media affecting the regeneration process enabled us to develop a technique to

obtain a reliable regeneration system with cucumber cotyledon and unexpanded leaf-derived callus using different genotypes.

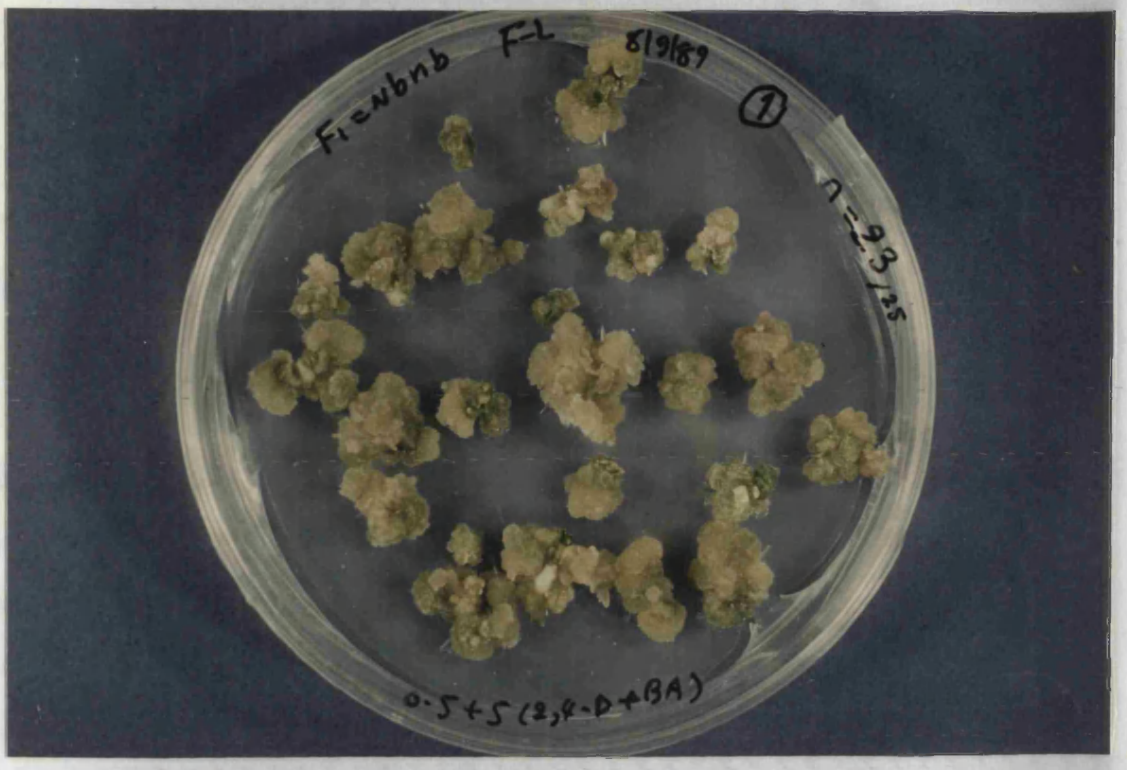
Cotyledon explants grown on media containing MS hormone free medium supplemented with 0.5 µM 2,4-D and 5 µM BA gave rise to organogensis calli that could be maintained through 2 to 3 successive transfer to the shoot regeneration media containing 0.5 µM NAA and 0.5 µM BA. The success in maintaining these regenerative calli are genotype-dependent. The highest number of shoots were obtained from genotypes Telegraph (Bookers seed Ltd) and telegraph (Sutton seeds Ltd), which the other investigators all are in general agreement in above results (Wehner & Locy, 1981; Trulson & Shahin, 1986; Kim et al, 1988). Thus the results underline the importance of choosing the proper genotype for in vitro studies.

It should be also point out that the probability of shoot regeneration via organogenesis or embryogenesis depends on callus induction or shoot regeneration media, in other words to auxin: cytokinin ratio. For instance in the case of Trulson & Shahin (1986) the regeneration was through the embryogenesis passway, but those of Wehner & Locy, Kim et al (1988) and also the author's was via organogenesis (Plate 38). Jelaska (1974) working with pumpkin reported similar results.

Due to the supply of limited seed for F_1 genotype (Nbnb) having bitter trait and abundance leaf material available from the regenerated shoots, this material was found to be an ideal source for explant.

Plate 37 (Top). Green islands initiation after two weeks in shoot regeneration media containing 0.25 μM BA which is followed by organogenesis (see Plate 38). x 1.2

Plate 38 (Bottom). Shoot regeneration on the media containing 0.25 μM BA. x 1.6


F1 cvbnb F-L 8/9/89
1
0.5+5 (2,4-D+BA)

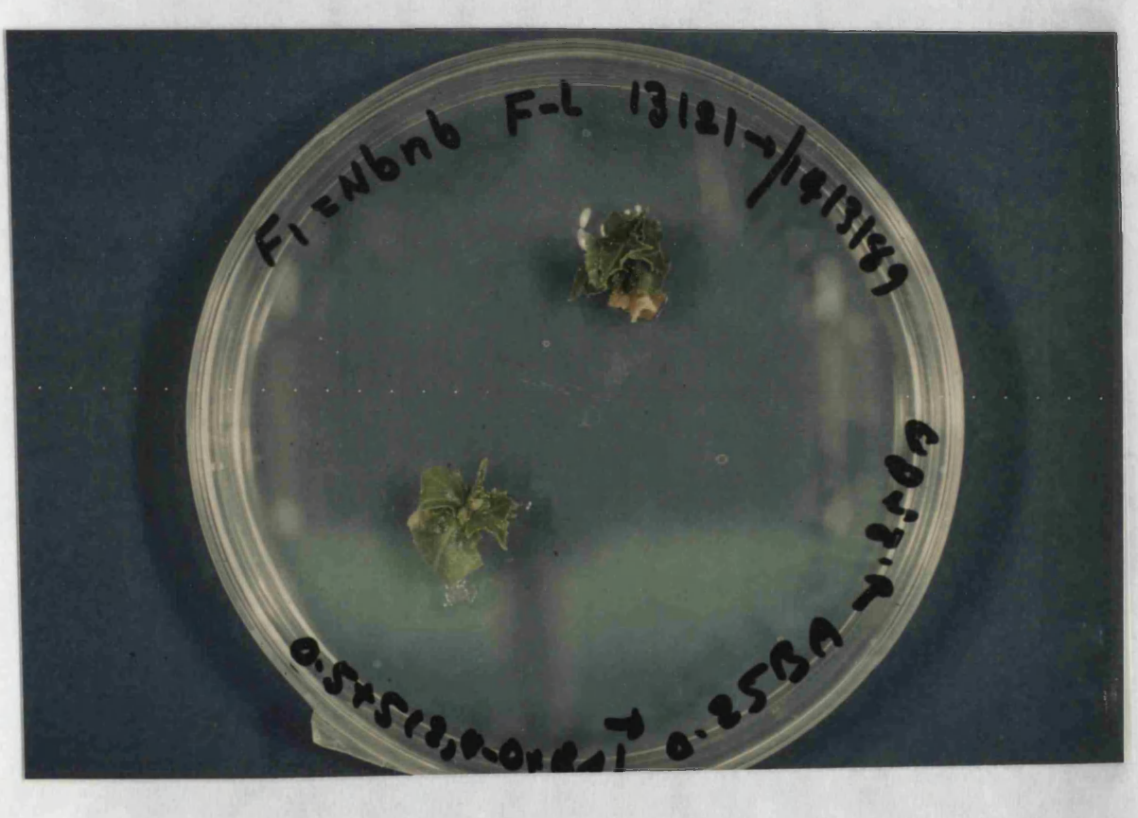

8/9/89 F1 ST-21

Although there were a few reports in the area of regeneration from leaf-derived callus in cucumber (Malepszy & Nadolska-orczyk, 1983; Nadolska-Orczyk & Malepszy, 1984; Nadolska-Orczyk, 1987; Malepszy & Nadolska-Orczyk, 1989). The unexpanded leaves were examined with different shoot regeneration media.

The highest regeneration frequencies were obtained on unexpanded leaf-derived callus on the media containing MS hormone free medium plus 0.25 μM BA (Table 32). Shoot formation also occurred on the callus induction media, but the shoots were not good in quality (Plate 35). The R_2 plantlets from the R_1 plants leaf-derived callus gave rise the minimum yield of shoots, these calli lost their shoot regeneration capacity in longer culture period, therefore the media containing the basal medium supplemented with 0.25 μM BA proved to be a good base to test the stability of bitterness trait in plantlets. All shoots produced in these experiments were through the adventitious organogenesis. Previous success of regeneration in cucumber (Malepszy & Nadolska-Orczyk, 1983; Nadolska-Orczyk & Malepszy, 1984; Nadolska-Orczyk, 1987; Malepszy & Nadolska-Orczyk, 1989) were obtained through the embryogenesis pathway.

The high frequency regeneration methods makes it possible to attempt to assess the stability of the agronomical important single-gene controlled trait of bitterness. Under regeneration conditions the results again indicated that 30°C was an affective regime for avoiding transition to reproductive phase since transition to reproductive phase occurs very early in 25°C regime (Plate 39).

Plate 39. Premature transition to reproductive stage on the shoot regeneration media, 16 hr photoperiod, 25°C.



In this work 747 R_1 regenerants have been regenerated, of which 11 were slightly bitter. Also 115 R_2 regenerants were regenerated by the same method, of which 2 were slightly bitter. Approximately 1-2% slightly bitter seedlings were found by Andeweg & De Bruyn, 1959 (Table,1) where, out of 11827 seedlings, 155 (1-2%) were slightly bitter and the rest were all bitter.

In tomato (Evans & Sharp, 1983) regeneration from leaves in vitro produced 13 independent mutations expressed at all developmental stages among 230 regenerated plants (one mutant out of 18). Assuming that the nuclear genome of higher plants consists of about 70000 structural gene (Goldberg, 1986) the mutation frequency in culture would be $(\frac{1}{18}\times\frac{1}{70000})$ or one gene in every 1260000 . Alternatively, the frequency of mutations in any one gene would also be 1 in 1.26×10^6, assuming the mutation frequency of all structural genes to be the same. Since the number of plants that could be regenerated in these experiments was smaller than 1.26×10^6 by about three orders of magnitude, it is not surprising that no non-bitter mutant phenotypes were recorded in this experiment.

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APPENDICES

APPENDIX 1

a. Formulation for Murashige & Skoog media (1962).

Substance	Amount in stock soln.	Stock soln. conc. mM	Dilution rate	medium conc. M
1. Macro Inorg.	g/litre			
KNo_3	20.20	2×10^{-1}	1: 10	2×10^{-2}
NH_4No_3	16.01	2×10^{-1}	1: 10	2×10^{-2}
$MgSo_4.7H_2o$	3.70	1.5×10^{-2}	1: 10	1.5×10^{-3}
KH_2Po_4	1.70	1.25×10^{-2}	1: 10	1.25×10^{-1}
$2.CaCl2.2H2o$	4.40	$3 \times 10\text{-}2$	1: 10	3×10^{-3}
3.Micro Inorg.	2mg/250ml			
H_3Bo_3	154.5	1×10^{-2}	1 : 100	1×10^{-4}
$MnSo_4.4H_2o$	557.75	1×10^{-2}	1 : 100	1×10^{-4}
$ZnSo_4.7H_2o$	215.50	3×10^{-3}	1 : 100	3×10^{-5}
$4.Na_2EDTA.2H_2o$	930.75	1×10^{-2}	1 : 100	1×10^{-4}
$FeSo_4.7H_2o$	795.50	1×10^{-2}	1 : 100	1×10^{-4}
5. Micro Organ.				
Myoinositol	2500.0	5×10^{-2}	1 : 100	5×10^{-4}
Glycine	47	2.5×10^{-3}	1 : 100	2.5×10^{-4}
6. Nano Inorg.	mg/100 ml			
$Na_2Moo_4.2H_2o$	24.2	1×10^{-3}	1 : 1000	1×10^{-6}
7. $CuSo_4.5\ H_2o$	2.5	1×10^{-4}	1 : 1000	1×10^{-7}
8. $CoCl_2.6H_2o$	2.5	1×10^{-4}	1 : 1000	1×10^{-7}
KI	83	5×10^{-3}	1 : 1000	5×10^{-6}
9. Vitamins				
Nicotinic acid	49.2	4×10^{-3}	1 : 1000	4×10^{-6}
Pyridoxine HCl	51.4	2.5×10^{-3}	1 : 1000	2.5×10^{-6}
Thiamine HCl	10.1	3×10^{-4}	1 : 1000	3×10^{-7}

b. Details of procedure for fixing, embedding, sectioninig and staining of unexpanded leaves.

1. Fix specimens in FAA (see below) for at least 18 hrs.

FAA fixative:	glacial acetic acid 5 ml	
	formalin 5 ml	
	ethanol (70%)	90 ml

2. Pass specimens through TBA series (see below) as follows:

TBA	Water	Ethanol (95%)	TBA	Ethanol (100%)
1	50	40	10	0.0
2	30	50	20	0.0
3	15	50	35	0.0
4	0.0	50	50	0.0
5	0.0	0.0	75	25

a. 50% ethanol 1 hr

b. TBA1 1 hr

c. TBA2 1 hr

d. TBA3 overnight

e. TBA4 1 hr

f. TBA5 1 hr

3. Transfer specimens to saturated solution of Erythrosin B made up pure TBA and leave

for one day on oven top at melting point of TBA (60-80°C).

4. Change to pure TBA and leave overnight on oven top.

5. Add wax chips to specimens in TBA. Put wax in vials till full and leave overnight inside the oven.

6. Remove wax and replace with fresh molten wax four times, leave for 3-4 hrs per wax change.

7. Embed the specimens in wax of a boat (8 x 4 x 1.5 cm) and orientt them with forceps. Allow wax to cool and immerse in cold water.

8. Section specimens at 6 um with micotome.

9. Make ribbon. Float sections on warm water to allow expansion and place on slide with thin smear of Haupt's adhesive (see below). Add a few drops of 3% formalin to allow further expansion.

Haupt's adhesive:

Dissolve 1 g gelatin in 100 ml distilled water at 60°C. Add 2 g phenol crystals and 15 ml og glycerol. Stir and filter.

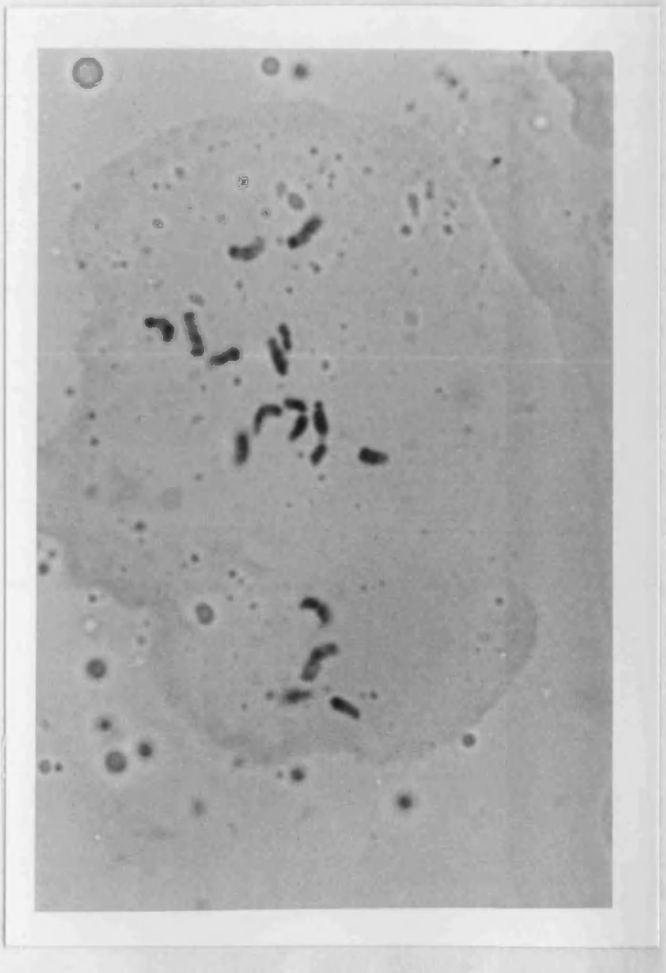
Staining procedures

		Time (min)
1. Immerse the basket of slides in xylene bath		3
2.		3
3.		3
4.	xylen/ethanole (50:50)	3
5.	ethanole (100%)	3
6.	ethanole (95%)	3
7.	ethanole (70%)	3
8.	ethanole (50%)	3

9.	ethanole (30%)	3
10.	Tap water	1
11.	stain	2
12.	stain	2
13.	Tap water	1
14.	ethanole (30%)	1
15.	ethanole (50%)	1/2
16.	ethanole (70%)	1/2
17.	ethanole (95%)	1/2
18.	ethanole (100%)	1/2
19.	xylene/ethanole (50:50)	1
20.	xylene	3
21.	xylene	3
22.	xylene	3
23.	xylene	3
24.	xylene	3

25. Attach cover slip to specimens on the slide with Canada Balsam.

c. Plate 40. Root tip methaphase chromosome of cucumber seedling. 2n=14. x 2681



d. Plates 41. Histological investigations provided evidence for direct differentiation of shoot bud fromunexpanded leaf (Maximum size, 3mm). Increasing magnification of LS of buds (right to left). Notice lack of vascular connection between bud and petiol's base. a= x 413 b= x 1040 c= x 1733 d= x 1052

APPENDIX 2.

a. Tables.

Table 2: Effects of hormone treatments on propagule numbers and shoot lengths from shoot tips of cv. Telegraph.

N_0= no of propagules at t=0

N_t= no of propagules after 32 days

Treatment number	N_0	Hormone concentration μ M	Ranking by shoot quality	$\frac{N_t}{N_0}$ ± S.E.M	Mean shoot length(cm) ± S.E.M
1	12	0.6 BA	12	6.3 ±0.8 a	2.7 ±0.5
2	7	0.3 NAA	7	5.5 ±0.8 a	2.9 ±0.6
3	9	0.3 NAA+ 0.6 BA	11	5.5 ±0.9 a	3.1 ± 0.5
4	9	0.4 BA	2	5.1 ±0.7 b	2.6 ± 0.6
5	7	0.4 NAA+ 0.3 BA	5	4.9 ±0.5 b	2.4 ± 0.1
6	8	0.3 NAA+ 0.4 BA	3	4.8 ±0.4 b	3.2 ± 0.9
7	7	0.3 BA	9	4.8 ±0.5 b	2.1 ± 0.3
8	10	0.6 NAA+ 0.4 BA	10	4.8 ±0.4 b	3.3 ± 0.8
9	7	0.3 NAA+ 0.3 BA	4	4.2 ± 0.3 b	2.8 ± 0.2
10	7	0.4 NAA	8	4.0 ±0.6 c	2.2 ± 0.8
11	10	0.4 NAA+0.6 BA	13	3.9 ± 0.4 c	2.1 ± 0.4
12	8	0.4 NAA+ 0.4 BA	6	3.6 ± 0.5 c	2.1 ± 0.2
13	10	Hormone-Free	1	3.6 ± 0.4 c	3.8 ± 0.7
14	10	0.6 NAA+ 0.6 BA	15	3.6 ± 0.5 c	1.7 ± 0.3
15	10	0.6 NAA+0.3 BA	16	3.2 ± 0.4 c	1.5 ± 0.2
16	10	0.6 NAA	14	2.5 ±0.4 d	2.7 ± 0.5

(There were no significant differences at the 1% level between treatments indicated with the same letter).

Table 3: Effects of hormone treatments on propagule numbers

and shoot lengths from shoot tips of cv. Rebella.

N_0= no of propagules at t=0

N_t= no of propagules after 32 days

Treatment number	N_0	Hormone concentration μ M	Ranking by shoot quality	$\frac{N_t}{N_0}$ ± S.E.M	Mean shoot length(cm) ± S.E.M
1	10	0.6 BA	10	5.7 ±0.4 a	3.9 ±0.4 a
2	9	0.3 NAA + 0.6 BA	15	5.3 ±0.6 a	3.6±0.2 a
3	8	0.3 NAA	5	5.3 ±0.5 a	3.1 ± 0.4 a
4	10	0.4 NAA + 0.4 BA	8	5.2 ±0.5 a	2.1 ± 0.4 c
5	12	0.4 BA	2	4.9 ±0.5 a	2.6 ± 0.4 b
6	13	0.3 NAA + 0.3 BA	3	4.8 ±0.5 a	2.5 ± 0.5 b
7	13	0.3 BA	6	4.8 ±0.4 a	2.0 ± 0.2 c
8	10	0.4 NAA + 0.6 BA	14	4.4 ±0.4 a	2.8 ± 0.3 a
9	11	Hormone-Free	1	4.3 ± 0.3 b	3.4 ± 0.7 a
10	11	0.6 NAA + 0.4 BA	12	4.0 ±0.8 b	2.7 ± 0.5 b
11	11	0.6 NAA + 0.6 BA	16	3.5 ± 0.4 b	2.4 ± 0.4 b
12	11	0.3 NAA + 0.4 BA	9	3.2 ± 0.5 c	1.3 ± 0.2 d
13	10	0.6 NAA	11	3.1 ± 0.3 c	3.2 ± 0.7 a
14	13	0.4 NAA	7	3.0 ± 0.3 c	1.9 ± 0.3 d
15	12	0.4 NAA + 0.3 BA	4	2.9 ± 0.3 c	1.6 ± 0.3 c
16	10	0.6 NAA + 0.3 BA	13	2.9 ±0.2 c	2.6 ± 0.4 b

(There were no significant differences at the 1% level between treatments indicated with the same letter).

Table 4: Effects of hormone treatments on propagule numbers

and shoot lengths from shoot tips of cv. Pepinex.

N_0= no of propagules at t=0

N_t= no of propagules after 32 days

Treatment number	N_0	Hormone concentration μ M	Ranking by shoot quality	$\frac{N_t}{N_0}$ ± S.E.M	Mean shoot length(cm) ± S.E.M
1	12	0.4 BA	2	8.3 ±0.7 a	2.7 ±0.4 b
2	10	0.6 BA	10	7.1 ±0.5 b	3.8 ±0.4 a
3	12	0.3 NAA + 0.4 BA	7	6.4 ±1.4 b	2.0 ± 0.3 b
4	11	0.6 NAA + 0.3 BA	12	6.3 ±0.5 b	4.7 ± 0.7 a
5	10	0.4 NAA + 0.6 BA	14	5.8 ±0.4 b	2.8 ± 0.2 b
6	10	0.3 NAA	3	5.7 ±0.3 b	3.6 ± 0.5 a
7	14	0.6 BA + 0.4 BA	13	5.6 ±0.4 b	3.9 ± 0.4 a
8	10	0.4 NAA + 0.4 BA	8	5.4 ±0.6 b	1.7 ± 0.3 b
9	7	0.6 NAA + 0.6 BA	15	5.4 ± 0.8 b	2.4 ± 0.3 b
10	10	0.3 NAA + 0.6 BA	16	5.4 ±0.6 b	2.3 ± 0.2 b
11	9	0.4 NAA	9	5.0 ± 0.7 b	2.6 ± 0.5 b
12	10	0.6 NAA	11	5.0 ± 0.4 c	5.2 ± 1.1 a
13	11	0.3 NAA + 0.3 BA	5	4.7 ± 0.4 c	1.9 ± 0.3 b
14	10	0.3 BA	4	4.5 ± 0.9 c	1.8 ± 0.3 b
15	7	Hormone-Free	1	4.4 ± 0.3 c	4.8 ± 0.9 a
16	7	0.4 NAA + 0.3 BA	6	4.4 ±1.4 d	1.6 ± 0.4 b

(There were no significant differences at the 1% level between treatments indicated with the same letter).

Table 9: The effect of hormone treatments on root induction and callusing over a 32 days culture period of cv. Telegraph

n_0= number of explants at t=0

Treatment number	n_0	Hormone concentration µ M	Extent of root formation ±S.E.M	Extent of callusing ±S.E.M
1	12	0.6 BA	2.9±0.6 a	4.3± 0.3 a
2	7	0.3 NAA	2.0 ±0.5 a	4.3± 0.3 a
3	9	0.3 NAA + 0.6 BA	2.5 ± 0.6 a	3.8 ± 0.3 b
4	9	0.4 BA	3.1 ± 0.9 a	3.6 ± 0.9 b
5	7	0.4 NAA +0.3 BA	2.7 ± 0.0 a	5.0 ± 0.0 a
6	8	0.3 NAA + 0.4 BA	1.6 ± 0.5 a	4.3 ± 0.3 a
7	7	0.3 BA	0.6 ± 0.3 b	2.7 ± 0.3 b
8	10	0.6 NAA + 0.4 BA	2.6 ± 1.1 a	4.9 ± 0.1 a
9	7	0.3 NAA + 0.3 BA	2.5 ± 0.5 a	4.8 ± 0.2 a
10	7	0.4 NAA	1.6 ± 1.9 a	4.5 ± 0.6 a
11	10	0.4 NAA + 0.6 BA	1.5 ± 0.7 a	4.3 ± 0.4 a
12	8	0.4 NAA + 0.4 BA	1.3 ± 1.8 b	5.0 ± 0.0 a
13	10	Hormone-Free	2.3 ± 0.3 a	0.4 ± 0.1 c
14	10	0.6 NAA + 0.6 BA	0.5 ± 0.0 b	4.9 ± 0.1 a
15	10	0.6 NAA + 0.3 BA	1.4 ± 0.8 b	5.0 ± 0.0 a
16	10	0.6 NAA	2.8 ± 0.8 d	4.7 ± 0.2 a

(There were no significant differences at the 1% level between treatments indicated with the same letter).

Table 10. The effect of hormone treatments on root induction and callusing over a 32 days culture period of cv. Rebella

n_0=no of explants at t= 0

Treatment number	n_0	Hormone concentration μ M	Extent of root formation ±S.E.M	Extent of callusing ±S.E.M
1	10	0.6 BA	4.2± 0.2 a	4.0± 0.5 b
2	9	0.3 NAA+0.6 BA	2.9 ± 1.4 b	4.6± 0.5 a
3	8	0.3 NAA	2.8± 0.7 b	3.1± 0.8 b
4	10	0.4 NAA+0.4 BA	1.4± 0.6 c	4.0± 0.5 b
5	12	0.4 BA	2.3± 0.3 b	3.0± 0.5 b
6	13	0.3 NAA+0.3 BA	1.3± 0.5 c	3.9± 0.3 b
7	13	0.3 BA	2.3± 0.6 b	2.7± 0.4 b
8	10	0.4 NAA+0.6 BA	1.1± 0.4 c	3.2±0.4 b
9	11	Hormone-Free	2.1± 0.2 b	0.5±0.7 c
10	11	0.6 NAA+0.4 BA	0.9± 0.4 c	2.9±0.4 b
11	11	0.6 NAA+0.6 BA	1.1± 0.4 c	4.9± 0.1 a
12	11	0.3 NAA+0.4 BA	0.7± 0.5 d	4.4± 0.4 a
13	10	0.6 NAA	2.3± 0.8 b	4.0±0.5 b
14	13	0.4 NAA	3.5± 0.7 a	3.4± 0.3 b
15	12	0.4 NAA+0.3 BA	0.6± 0.3 d	2.9± 0.5 b
16	10	0.6 NAA+0.3 BA	0.6± 0.2 d	1.8± 0.1 b

(There were no significant differences at the 1% level between treatments indicated with the same letter).

Table 11. The Effect of hormone treatments on root induction and callusing over 32 days culture period of cv. Pepinex.

n_0= number of explants at t=0

Treatment number	n_0	Hormone concentration μ M	Extent of root formation	Extent of callusing ± S.E.M
1	12	0.4 BA	1.8 ± 0.6	4.0 ± 0.4 b
2	10	0.6 BA	3.7 ± 0.3	4.7 ± 0.2 a
3	12	0.3 NAA + 0.4 BA	1.8 ± 0.4	4.3 ± 0.5 a
4	11	0.6 NAA + 0.3 BA	2.7 ± 0.5	4.9 ± 0.1 a
5	10	0.4 NAA + 0.6 BA	2.5 ± 0.5	5.0 ± 0.0 a
6	10	0.3 NAA	2.6 ± 0.4	2.4 ± 0.2 a
7	14	0.6 NAN + 0.4 BA	3.1 ± 0.5	4.9 ± 0.1 a
8	10	0.4 NAA + 0.4 BA	1.4 ± 0.5	3.6 ± 0.5 b
9	7	0.6 NAA + 0. 6 BA	1.9 ± 0.9	5.0 ± 0.0 a
10	10	0.3 NAA + 0.6 BA	1.0 ± 0.7	5.0 ± 0.0 a
11	9	0.4 NAA	3.0 ± 1.4	4.9 ± 0.1 a
12	10	0.6 NAA	2.7 ± 0.6	4.4 ± 0.3 a
13	11	0.3 NAA + 0.3 BA	2.3 ± 0.5	3.8 ± 0.5 b
14	10	0.3 BA	2.6 ± 0.7	3.6 ± 0.2 b
15	7	Hormoe-Free	2.4 ± 0.3	0.0 ± 0.0 c
16	7	0.4 NAA + 0.3 BA	0.8 ± 1.1	4.4 ± 0.7 d

(There were no significant differences at the 1% level between treatments indicated with the same letter).

Table 14: Effect of different temperatures on propagule numbers

after 33 days of culture on 0.35μ M BA

Temp (C^0) / Cultivars	20		25		30	
	N_0	Mean $\frac{N_t}{N_0}$ ± SEM	N_0	$Mean\frac{N_t}{N_0}$ ± SEM	N_0	$Mean\frac{N_t}{N_0}$ ± SEM
Telegraph	9	6.0 ± 0.5 b	11	6.2± 0.3 b	12	6.8± 0.4 a
Rebella	10	6.4± 0.3 b	10	7.7± 0.6 a	10	8.5± 0.5 a
Pepinex	9	5.7± 0.5 b	11	7.3±0.4 a	10	7.0± 0.6 a

(There were no significant differences at the 1% level between treatments indicated with the same letter).

Table 15: Mean shoot length (cm) in shoot tip cultures on 0.35μ M

BA, at different temperatures after 33 days of culture.

Temp (C^0) / Cultivars	20		25		30	
	N_0	Mean shoot length ± SEM	N_0	Mean shoot length ± SEM	N_0	Mean shoot length ± SEM
Telegraph	9	2.0 ± 0.2 d	11	4.8± 0.2 b	12	5.3± 0.3 a
Rebella	9	2.3± 0.2 d	10	3.7± 0.3 c	10	5.9± 0.8 a
Pepinex	9	2.5± 0.2 d	11	3.8±0.5 d	10	5.9± 0.4 a

(There were no significant differences at the 1% level between treatments indicated with the same letter).

Table 23. Composition of the basal medium for immature inflorescence culture.

Components	Stock solution	Concentration (g/l)	Amount/l (ml)	Final Conc/l
$Ca(No_3)_2.4\ H_2o$	I	8.0	25	200 mg
KNo_3	I	3.2		80 mg
$NaH_2Po_4.\ H_2o$	II	0.7	25	17.5 mg
Na_2HSo_4	II	8.0		200 mg
$MgSo_4.\ 7\ H_2o$	III	14.4	25	360 mg
KCl	III	2.6		65 mg
Ferric citrate	IV	2.0		2.0 mg
$MnSo_4.\ 4\ H_2o$	IV	4.5	1	4.5 mg
$ZnSo_4.\ 7\ H_20$	IV	1.5		1.5 mg
H_3Bo_3	IV	1.5		1.5 mg
Glycine	V	0.3		3.0 mg
Niacin	V	0.05/100 ml		0.5 mg
Pyridoxine	V	0.01/100 ml	1	0.1 mg
Thiamine	V	0.01/100 ml		0.1 mg
Tryptophan	V	0.5/100 ml		5.0 mg
Kinetin	VI	0.01/100 ml	1	0.1 mg
Casein hydrolyzate	VII	10%	2	0.02%
Coconut milk (CM)				150 ml
Sucrose				20 g
Agar				8 g

table 24. Effect of IAA concentrations on sex expression in immature inflorescences, after 2-3 months's of culture. Maximum flower bud size in explants:0.7 mm.

Sex expression / Genotypes / IAA conc(mg/l)		No of explants	No of flowers			
			Male	Female	Total	per explant
	0	24	144	0.0	144	6
F_1 (Tele/Chip)	0.2	26	174	0.0	174	7
	0.4	30	105	0.0	105	4
	0	19	151	1.0	152	8
F_1	0.2	15	143	0.0	143	10
(Perfection x	0.4	13	59	0.0	59	5
Ottawa)						
	0	10	78	0.0	78	8
P_1 (Perfection)	0.2	7	96	0.0	96	14
	0.4	8	87	0.0	87	11
	0	22	118	1.0	119	5
	0.2	18	253	0.0	253	14
P_2 (Ottawa)	0.4	19	234	1.0	235	12

Table 25. Effect of IAA concentrations on sex expression in immature inflorescences, after 90 days culture at 23°C day, 17°C night, 8 hr photoperiod. Maximum flower bud size in explants 0.7 mm.

Sex expression		No of explant	Number of flowers			
Genotypes / IAA conc (mg/l)			Male	Female	Total	flowers/ explant
	0	9	78	1.0	79	9
	0.1	12	168	0.0	168	14
P_2(Ottawa)	0.3	13	195	0.0	195	15
	1.0	18	182	0.0	182	10
F_1	0	9	111	0.0	111	12
(Perfec x	0.1	13	163	0.0	163	13
Ottawa)	0.3	15	210	0.0	210	15
	1.0	10	99	1.0	100	10
	0	12	166	0.0	166	14
P_1	0.1	11	163	0.0	163	15
(Perfectio	1.0	10	85	0.0	86	9
n)						
Telegraph	0	10	84	0.0	84	8
(Bookers)	1.0	13	128	0.0	128	10

Table 27. Effect of photoperiod on sex expression (mean of the three cultivars used in the experiment).

Sex expression	Male flowers		Female flowers		Total	
Photoperiod (hr)	No	%	No	%	No	%
8	290	97	10	3	300	100
12	176	65	94	35	270	100
16	92	59	65	41	157	100

Table 28. Effect of photoperiod on plant growth (mean of the three cultivars used in the experiment).

Plant growth Photoperiods (hr)	Fresh weight (g)	Shoot length (cm)	Propagule number (mean)
8	1.57	4.84	4.73
12	2.43	3.43	4.37
16	1.60	3.90	4.43

Table 29. Effect of photoperiod on sex expression of the three cultivars used in this experiment.

Sex expression / Cultivars	Photopds (hr)	Number of explants	Male		Female		Total	
			Total	%	Total	%		%
Telegraph	8	10	153	97	5	3	158	100
	12	10	176	90	20	10	196	100
	16	7	56	98	1	2	57	100
Rebella	8	9	29	85	5	15	34	100
	12	10	0.0	0.0	35	100	35	100
	16	10	0.0	0.0	38	100	38	100
Pepinex	8	9	108	100	0.0	0.0	108	100
	12	8	0.0	0.0	39	100	39	100
	16	11	36	68	26	32	62	100

Table 30. Effect of photoperiod on shoot growth after 57 day culture on MS hormone free medium, at 20°C in vitro.

Photoperiod/ Plant growth Cultivar	Photopd (hr)	No of explant	Fresh weight mean± sem (g)	Shoot length mean± sem (cm)	No of propagule mean± sem
	8	10	1.4± 0.2	3.9± 0.6	4.5± 0.5
Telegraph	12	10	2.3± 0.5	3.4± 0.5	4.7± 0.5
	16	7	1.6± 0.2	3.5± 0.3	4.1± 0.3
	8	9	2.0± 0.3	4.8± 1.1	5.5± 0.5
Rebella	12	10	2.3± 0.5	3.0± 0.3	3.8± 0.4
	16	10	1.6± 0.1	3.6± 0.3	4.6± 0.3
	8	9	1.3± 0.3	5.9± 0.9	4.2± 0.3
Pepinex	12	8	2.7± 0.4	3.9± 0.5	4.6± 0.3
	16	11	1.6± 0.2	4.6± 0.3	4.6± 0.3

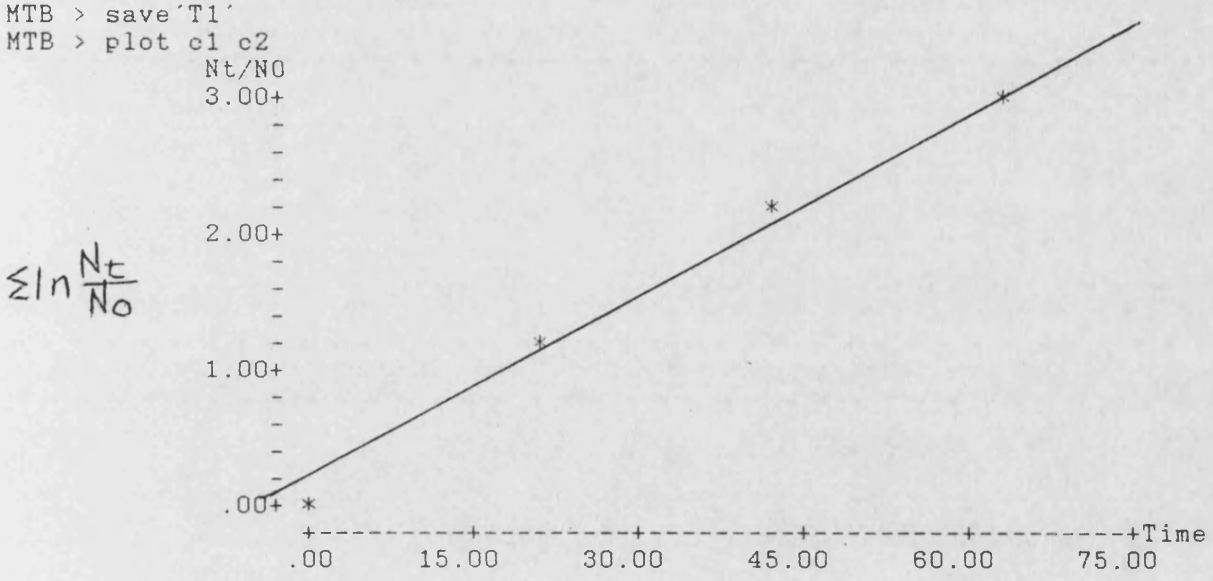
Table 31. Shoot regeneration from cotyledon-derived callus after 2 months culture (25°C,16 hr photoperiod) on callus induction medium : 0.5 µM 2,4-D + 5.0 µM BA and shoot Regeneration medium: 0.5 µM NAA + 0.5 µM BA

Regeneration incidence / Genotypes	Original explant number	No of responding explants	Explant response	Explant productivity	Notes
Telegraph (Bookers seed Ltd)	22	6.0	0.27	2.33	compact callus
Telegraph (Sutton seed Ltd)	20	9.0	0.45	1.33	compact callus
P_1(Telegraph)	6	0.0	0.0	0.0	no calli
P_2(Chipper)	6	0.0	0.0	0.0	green
F_1(Tele x Chip)	6	2.0	0.33	1.0	green
P_1(NbNb)bitter	8	2.0	0.25	1.0	green
P_2(nbnb)non-bit	4	1.0	0.25	1.0	green
F_1 (Nbnb)	6	1.0	0.17	1.0	green
Rebella	26	1.0	0.04	1.0	friable
Pepinex	27	3.0	0.11	1.33	green

b. Figures.

Fig 10. Regression analysis of the log of the proliferation ratio on time of culture cv. Telegraph on 0.3 µM NAA
Explant : shoot tip

```
MTB > save'T1'
MTB > plot c1 c2
```



$\Sigma \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = 0.132 + 0.0468 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
                 0.1320         0.1750          0.75
Time          0.046762       0.004454         10.50

S = 0.2091

R-SQUARED = 98.2 PERCENT
R-SQUARED = 97.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       4.8216           4.8216
RESIDUAL      2       0.0875           0.0437
TOTAL         3       4.9091


DURBIN-WATSON STATISTIC = 2.05

MTB >
```

log of the

Fig 11. Regression analysis of the proliferation ratio on time of culture cv. Telegraph on 0.3 μM NAA Explant : node 1

```
MTB > save'T2'
MTB > plot c1 c2
                C1
           2.40+
               -
               -
               -
               -
           1.60+
               -
               -
               -
               -
            .80+
               -
               -
               -
               -
            .00+ *
               +---------+---------+---------+---------+---------+---------+Time
              .00      15.00     30.00     45.00     60.00     75.00
```

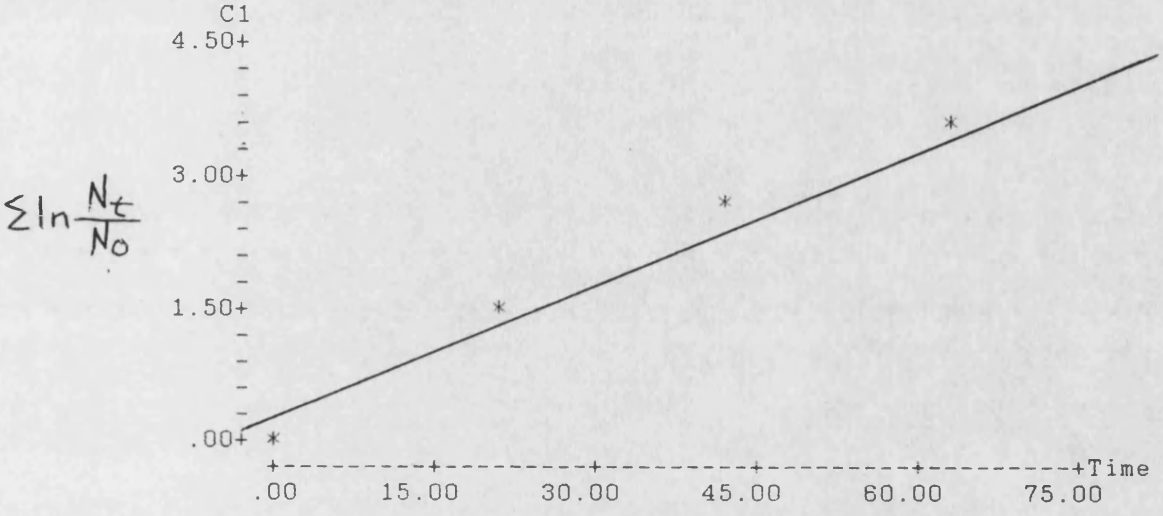
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = 0.0660 + 0.0270 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              0.06600           0.08350          0.79
Time         0.026952          0.002125         12.68

S = 0.09980

R-SQUARED = 98.8 PERCENT
R-SQUARED = 98.2 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        1.6018          1.6018
RESIDUAL      2        0.0199          0.0100
TOTAL         3        1.6217


DURBIN-WATSON STATISTIC = 2.02

MTB >
```

log of the

Fig 12. Regression analysis of the proliferation ratio on time of culture cv. Telegraph on 0.3 µM NAA Explant : node 2

```
DATA> end
MTB > save'T3'
MTB > plot c1 c2
```



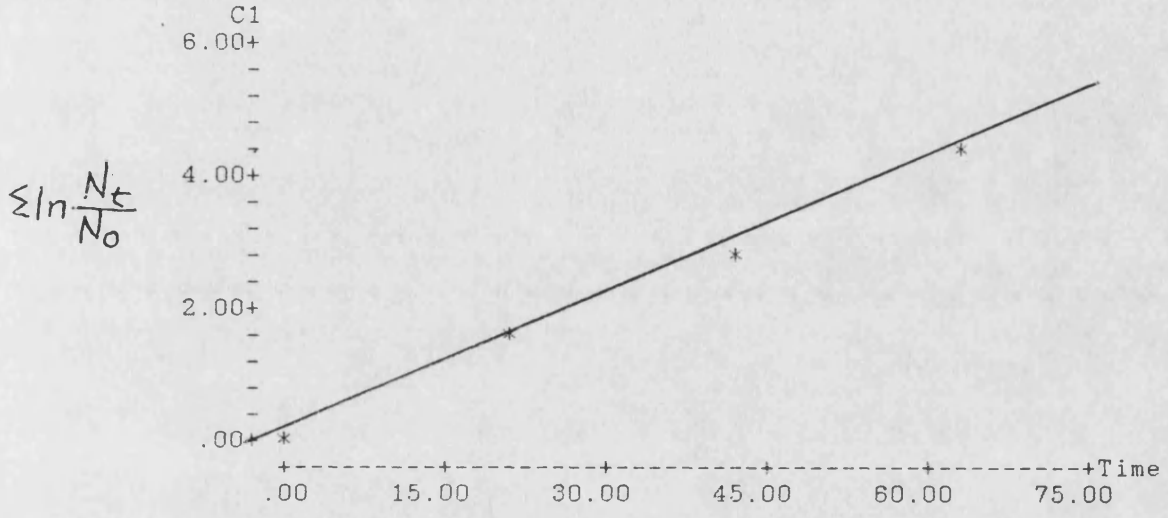
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = 0.110 + 0.0568 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
                 0.1100         0.1274          0.86
Time          0.056905       0.003242         17.55

S = 0.1522

R-SQUARED = 99.4 PERCENT
R-SQUARED = 99.0 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS        MS=SS/DF
REGRESSION    1       7.1401          7.1401
RESIDUAL      2       0.0463          0.0232
TOTAL         3       7.1865


DURBIN-WATSON STATISTIC = 2.00

MTB >
```

log of the

Fig 13. Regression analysis of the proliferation ratio on time of culture cv. Telegraph on 0.4 μ M BA Explant : shoot tip

```
DATA> end
MTB > save'T4'
MTB > plot c1 c2
```



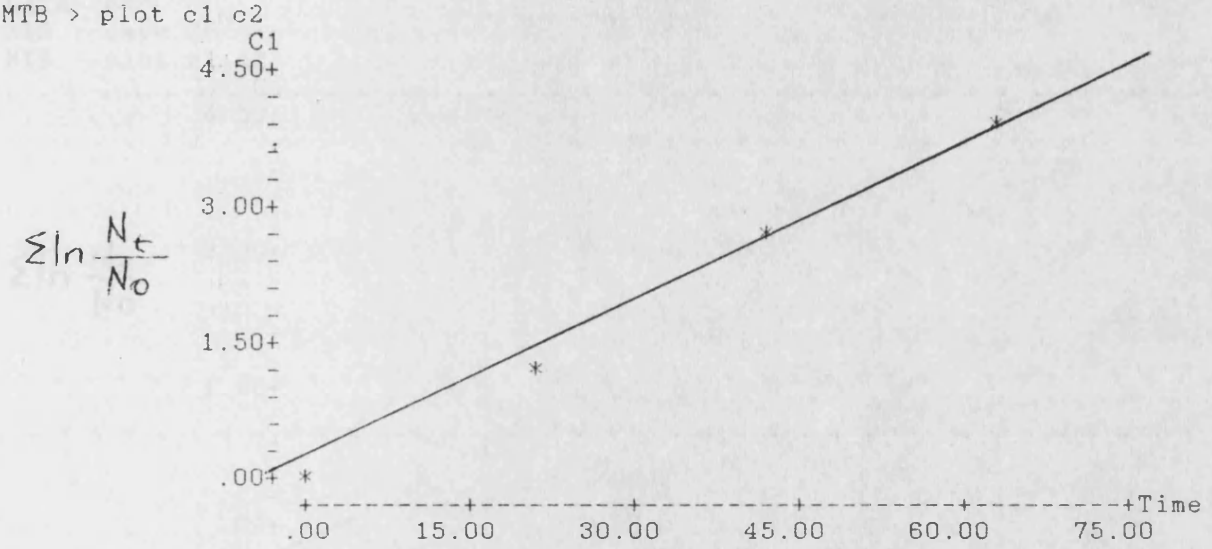
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.0200 + 0.0729 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.02000          0.03240         -0.62
Time         0.0728571        0.0008248         88.33

S = 0.03873

R-SQUARED =100.0 PERCENT
R-SQUARED =100.0 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1       11.704            11.704
RESIDUAL      2        0.003             0.001
TOTAL         3       11.707


DURBIN-WATSON STATISTIC = 2.23

MTB >
```

log of the

Fig 14. Regression analysis of the proliferation ratio on time of culture cv. Telegraph on 0.4 μ M BA Explant : node 1

```
DATA> end
MTB > save'T5'
MTB > plot c1 c2
```



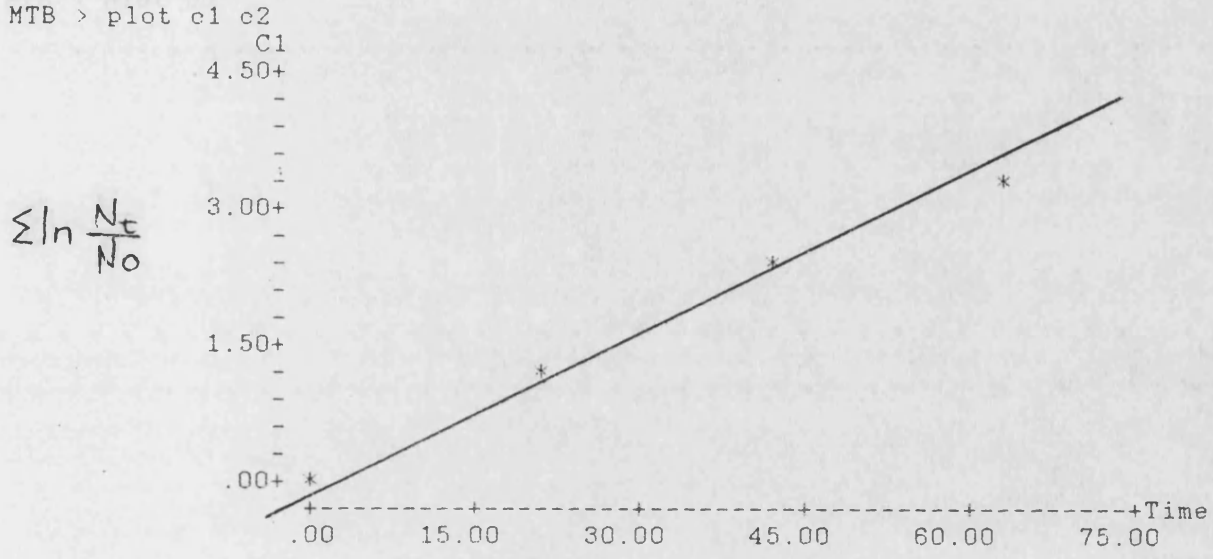
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = 0.0160 + 0.0608 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              0.01600           0.05130          0.31
Time         0.060762          0.001306         46.53

S = 0.06132

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO     DF            SS         MS=SS/DF
REGRESSION   1         8.1409           8.1409
RESIDUAL     2         0.0075           0.0038
TOTAL        3         8.1484


DURBIN-WATSON STATISTIC = 2.73

MTB >
```

log of the

Fig 15. Regression analysis of the proliferation ratio on time of culture cv. Telegraph on 0.4 μM BA Explants: node 2

```
DATA> end
MTB > save'T6'
MTB > plot c1 c2
```

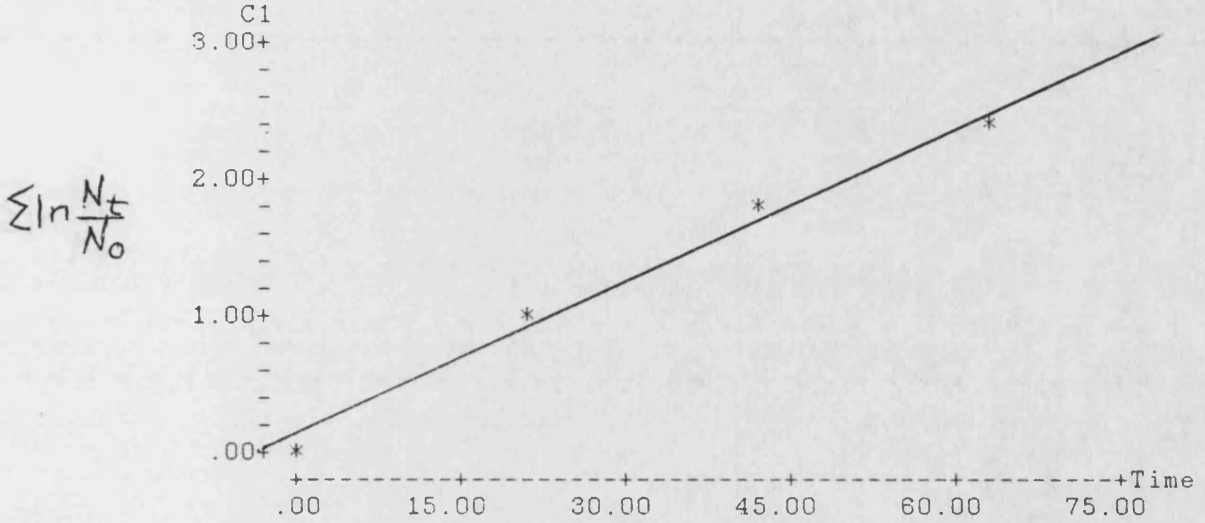


$\Sigma \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.0360 + 0.0550 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
             -0.03600           0.04150         -0.87
Time          0.054952          0.001056        52.03

S = 0.04960

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        6.6586          6.6586
RESIDUAL      2        0.0049          0.0025
TOTAL         3        6.6635


DURBIN-WATSON STATISTIC = 2.69

MTB >
```

Fig 16. Regression analysis of the log of the proliferation ratio on time of culture cv. Rebelia on 0.3 μM NAA Explant : shoot tip

```
DATA> end
MTB > save'T7'
MTB > plot c1 c2
```



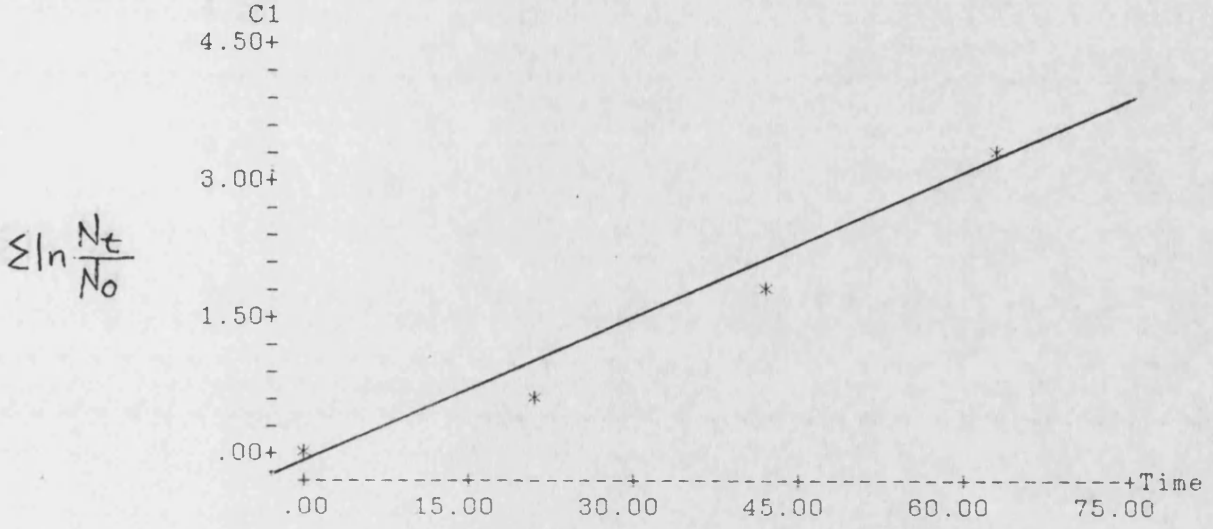
$\sum \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = 0.0550 + 0.0388 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
              0.05500         0.06836          0.80
Time          0.038810        0.001740        22.31

S = 0.08170

R-SQUARED = 99.6 PERCENT
R-SQUARED = 99.4 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        3.3211           3.3211
RESIDUAL      2        0.0133           0.0067
TOTAL         3        3.3345


DURBIN-WATSON STATISTIC = 2.01

MTB >
```

log of the

Fig 17. Regression analysis of the proliferation ratio on time of culture cv. Rebella on 0.3 µM NAA Explant : node 1

```
DATA> end
MTB > save'T8'
MTB > plot c1 c2
```

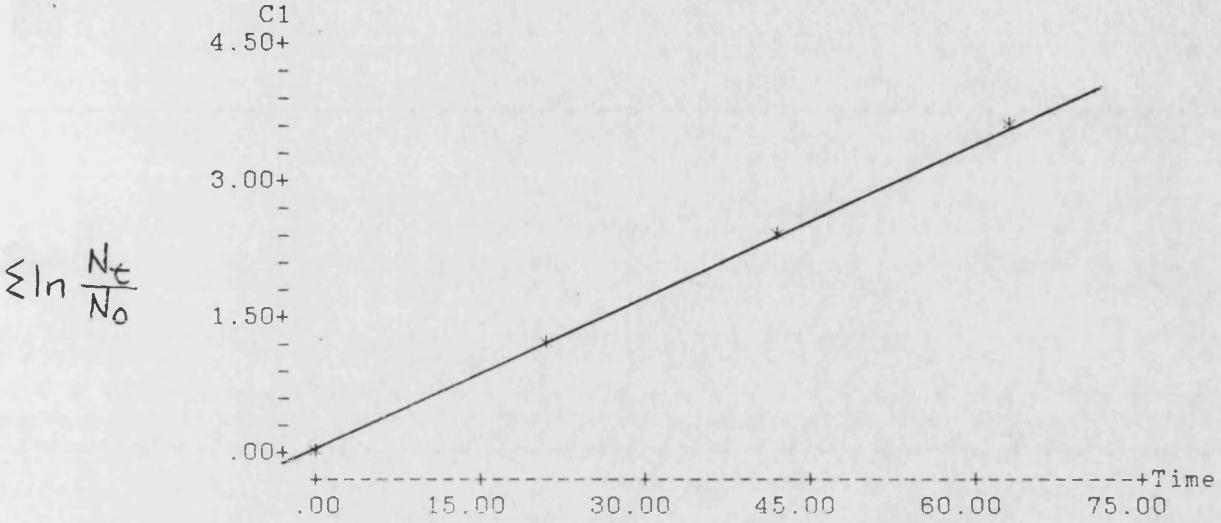


$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.147 + 0.0511 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.1470            0.1594         -0.92
Time          0.051095         0.004058         12.59

S = 0.1906

R-SQUARED = 98.8 PERCENT
R-SQUARED = 98.1 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       5.7566           5.7566
RESIDUAL      2       0.0726           0.0363
TOTAL         3       5.8293


DURBIN-WATSON STATISTIC = 2.15

MTB >
```

Fig 18. Regression analysis of the log of the proliferation ratio on time of culture cv. Rebelia on 0.3 μM NAA
Explant : node 2

```
DATA> end
MTB > save'T9'
MTB > plot c1 c2
```

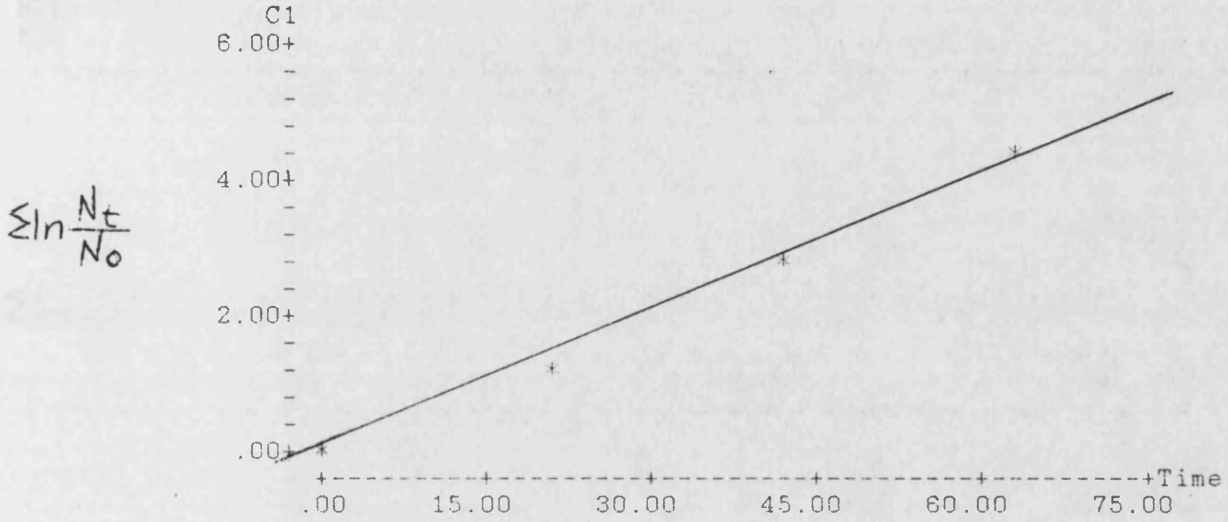



```
MTB >

THE REGRESSION EQUATION IS
C1 = 0.0260 + 0.0555 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
              0.02600         0.06010          0.43
Time          0.055524        0.001530        36.30

S = 0.07183

R-SQUARED = 99.8 PERCENT
R-SQUARED = 99.8 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        6.7978           6.7978
RESIDUAL      2        0.0103           0.0052
TOTAL         3        6.8081


DURBIN-WATSON STATISTIC = 2.53

MTB >
```

log of the

Fig 19. Regression analysis of the proliferation ratio on time of culture cv. Rebella on 0.4 μ M BA
Explant : shoot tip

```
DATA> end
MTB > save'T10'
MTB > plot c1 c2
```



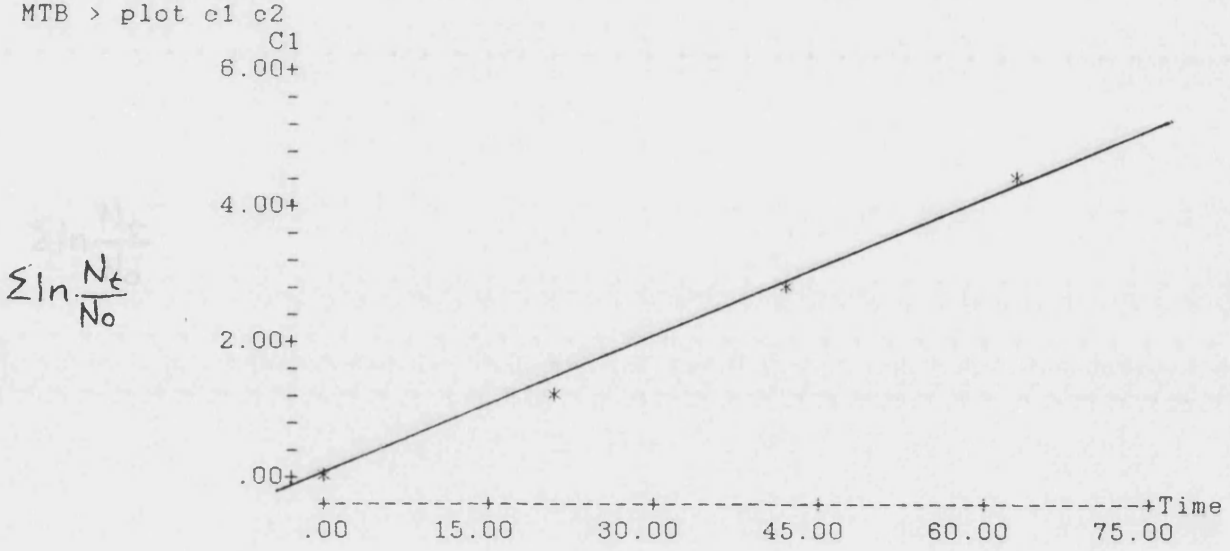
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.0400 + 0.0690 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
             -0.04000          0.04347         -0.92
Time          0.069048         0.001107        62.40

S = 0.05196

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1        10.513          10.513
RESIDUAL      2         0.005           0.003
TOTAL         3        10.518


DURBIN-WATSON STATISTIC = 2.13

MTB >
```

log of the

Fig 20. Regression analysis of the proliferation ratio on time of culture cv. Rebelia on 0.4 μ M BA Explant : node 1

```
DATA> end
MTB > save 'T11'
MTB > plot c1 c2
```



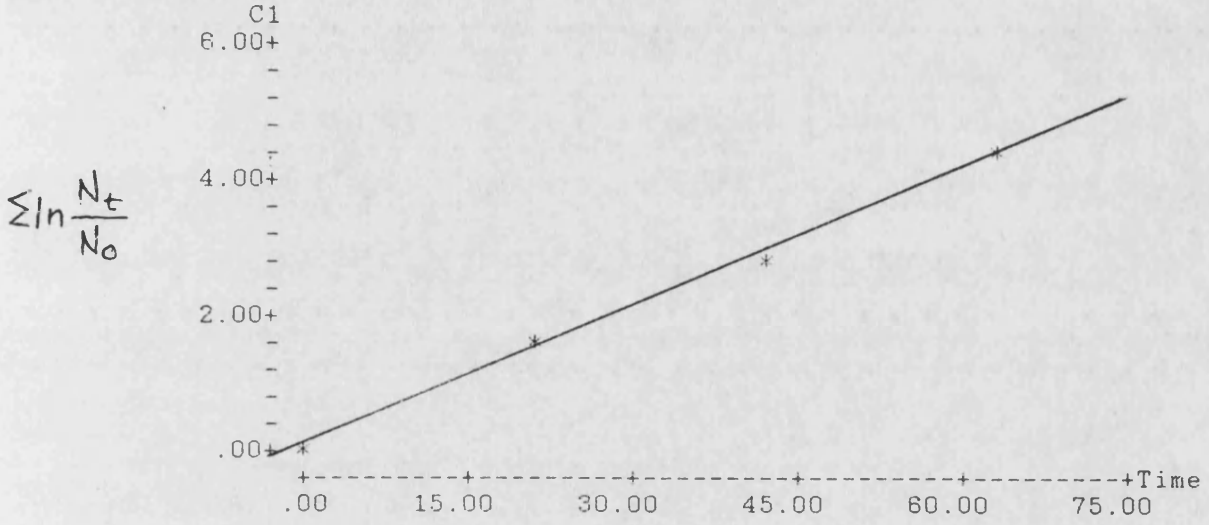
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.0380 + 0.0670 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
             -0.03800           0.04439        -0.86
Time          0.067000          0.001130       59.30

S = 0.05306

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        9.8982          9.8982
RESIDUAL      2        0.0056          0.0028
TOTAL         3        9.9039


DURBIN-WATSON STATISTIC = 2.00

MTB >
```

log of the

Fig 21. Regression analysis of the proliferation ratio on time of culture cv. Rebelia on 0.4 μ M BA Explant : node 2

```
DATA> end
MTB > save'T12'
MTB > plot c1 c2
```



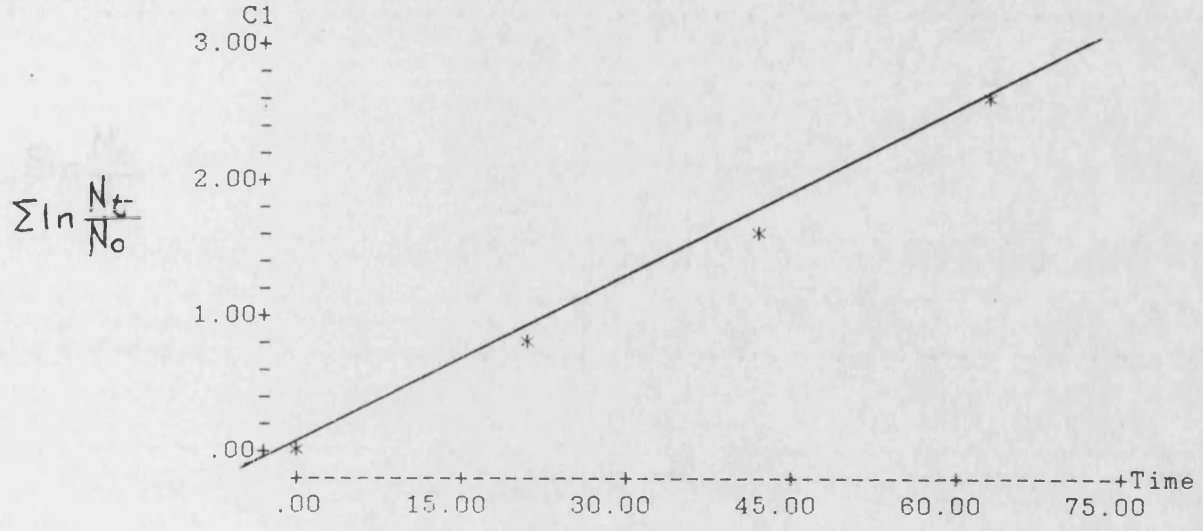
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.0220 + 0.0706 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.02200          0.02666        -0.83
Time         0.0706190        0.0006785        104.09

S = 0.03186

R-SQUARED =100.0 PERCENT
R-SQUARED =100.0 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       10.996           10.996
RESIDUAL      2        0.002            0.001
TOTAL         3       10.998


DURBIN-WATSON STATISTIC = 2.00

MTB >
```

log of the

Fig 22. Regression analysis of the proliferation ratio on time of culture cv. Pepinex on 0.3 μ M NAA Explant : shoot tip

```
DATA> end
MTB > save'T13'
MTB > plot c1 c2
```



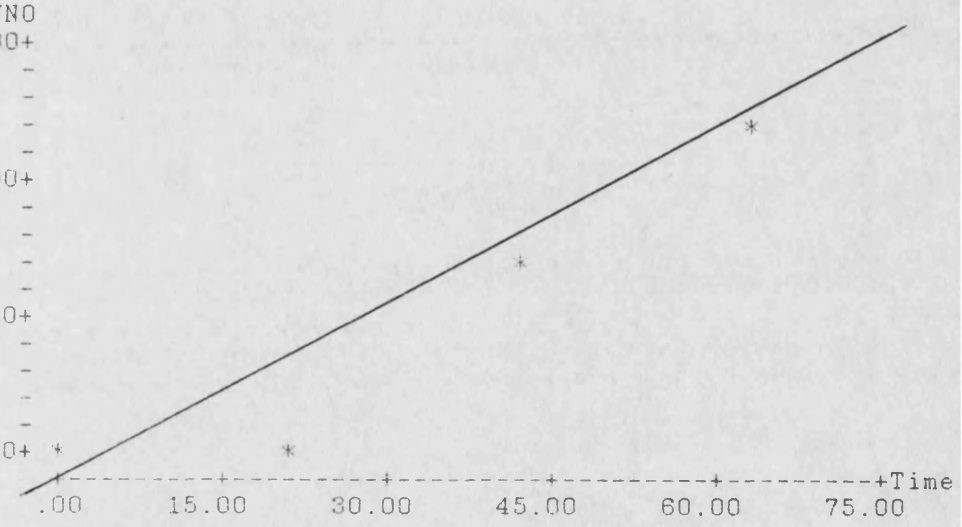
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.0520 + 0.0418 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
             -0.05200         0.07408         -0.70
Time          0.041810        0.001886        22.17

S = 0.08854

R-SQUARED = 99.6 PERCENT
R-SQUARED = 99.4 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        3.8544           3.8544
RESIDUAL      2        0.0157           0.0078
TOTAL         3        3.8701


DURBIN-WATSON STATISTIC = 2.11

MTB >
```

Fig 23. Regression analysis of the log of the proliferation ratio on time of culture cv. Pepinex on 0.3 μ M NAA
Explant : node 1

$\Sigma \ln \frac{N_t}{N_o}$

```
MTB > retr 'T14'
MTB > plot c1 c2
```

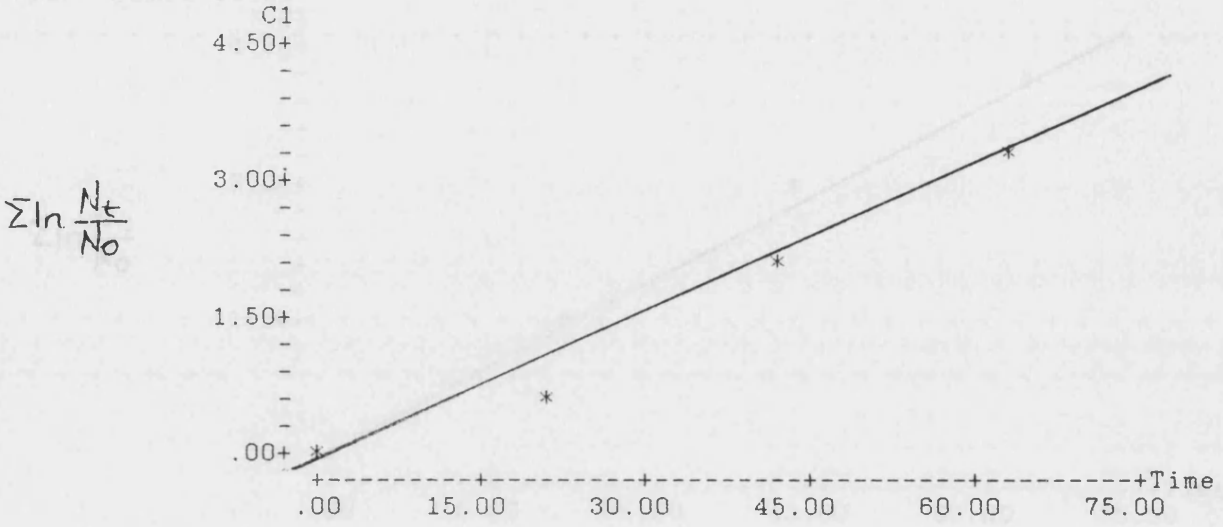



```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.340 + 0.0402 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.3400            0.3748         -0.91
Time         0.040238          0.009540          4.22

S = 0.4480

R-SQUARED = 89.9 PERCENT
R-SQUARED = 84.8 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS        MS=SS/DF
REGRESSION    1       3.5701          3.5701
RESIDUAL      2       0.4013          0.2007
TOTAL         3       3.9715


DURBIN-WATSON STATISTIC = 2.47

MTB >
```

log of the

Fig 24. Regression analysis of the proliferation ratio on time of culture cv. Peplnex on 0.3 μ M NAA Explant : node 2

```
DATA> end
MTB > save'T15'
MTB > plot c1 c2
```



$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.153 + 0.0515 Time
```

N_t/N_0

COLUMN	COEFFICIENT	ST. DEV. OF COEF.	T-RATIO = COEF/S.D.
	-0.1530	0.1728	-0.89
Time	0.051524	0.004400	11.71

```
S = 0.2066

R-SQUARED = 98.6 PERCENT
R-SQUARED = 97.8 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE
```

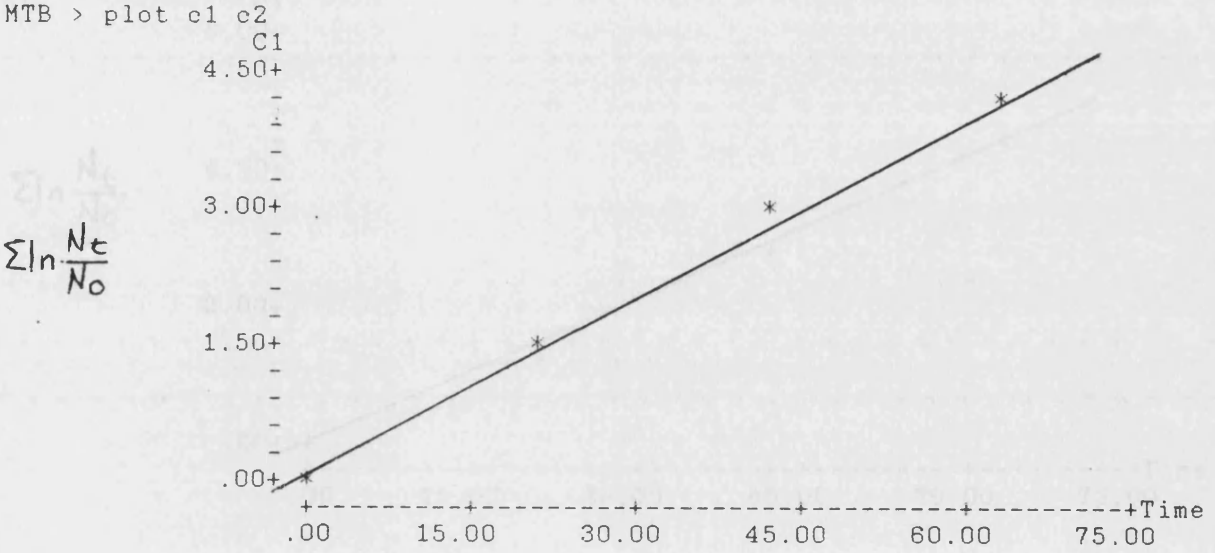
DUE TO	DF	SS	MS=SS/DF
REGRESSION	1	5.8536	5.8536
RESIDUAL	2	0.0854	0.0427
TOTAL	3	5.9390	

```
DURBIN-WATSON STATISTIC = 2.61

MTB >
```

log of the

Fig 25. Regression analysis of the proliferation ratio on time of culture cv. Pepinex on 0.4 μ M BA
Explant : shoot tip

```
DATA> end
MTB > save 'T16'
MTB > plot c1 c2
```



$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = 0.046 + 0.0658 Time
```

N_t / N_0

COLUMN	COEFFICIENT	ST. DEV. OF COEF.	T-RATIO = COEF/S.D.
	0.0460	0.1239	0.37
Time	0.065762	0.003154	20.85

```
S = 0.1481

R-SQUARED = 99.5 PERCENT
R-SQUARED = 99.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE
```

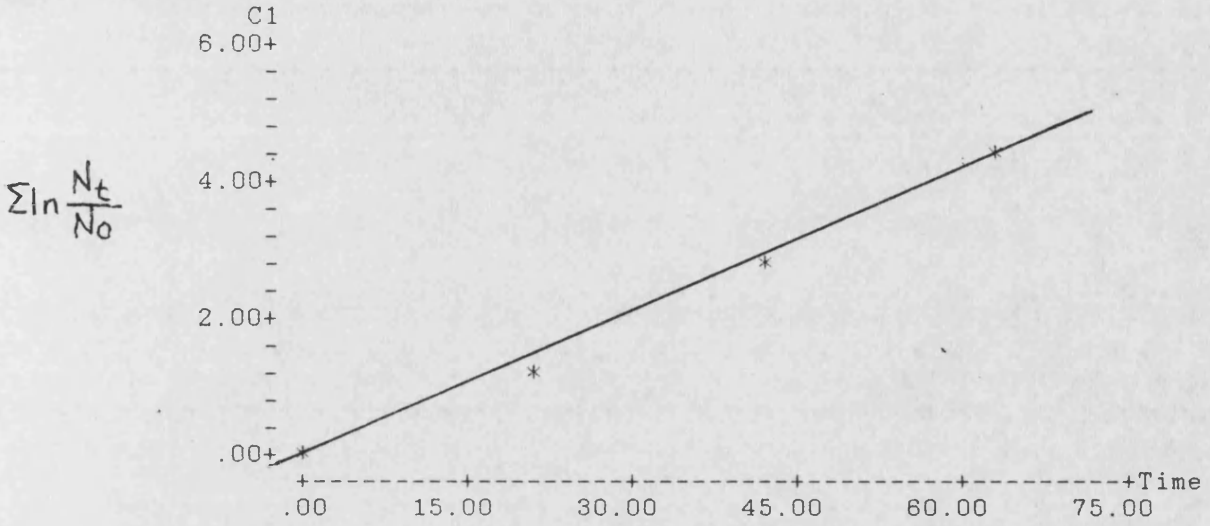
DUE TO	DF	SS	MS=SS/DF
REGRESSION	1	9.5358	9.5358
RESIDUAL	2	0.0439	0.0219
TOTAL	3	9.5797	

```
DURBIN-WATSON STATISTIC = 2.63

MTB >
```

log of the

Fig 26. Regression analysis of the proliferation ratio on time of culture cv. Pepinex on 0.4 μ M BA Explant : node 1

```
DATA> end
MTB > save'T17'
MTB > plot c1 c2
```

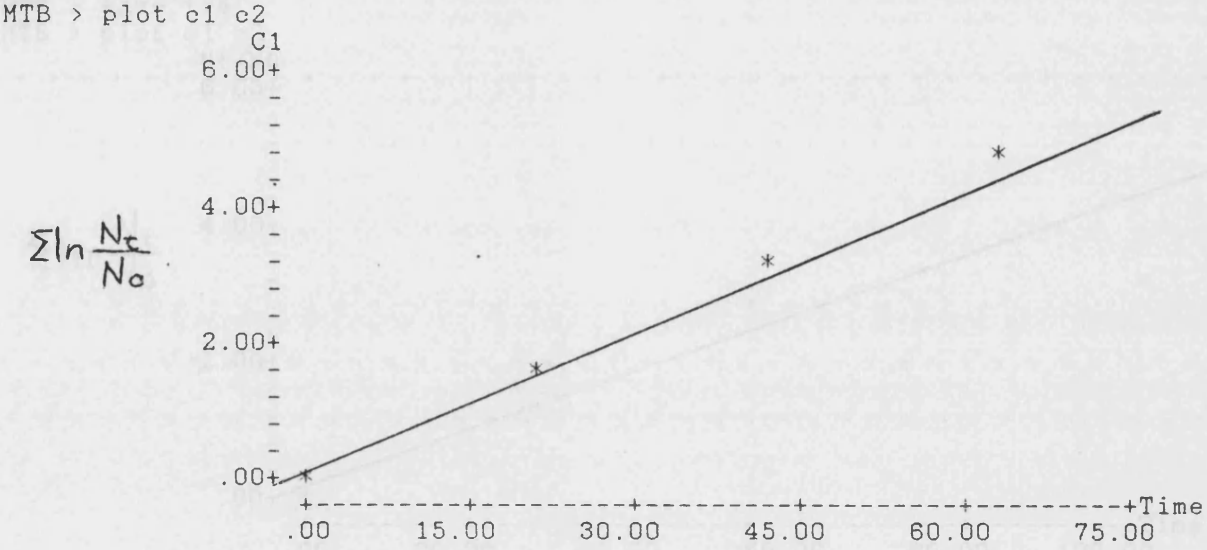


$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.0580 + 0.0725 Time
Nt/No

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.05800          0.06283         -0.92
Time          0.072476         0.001599         45.32

S = 0.07510

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS        MS=SS/DF
REGRESSION    1       11.582          11.582
RESIDUAL      2        0.011           0.006
TOTAL         3       11.594


DURBIN-WATSON STATISTIC = 2.16

MTB >
```

Fig 27. Regression analysis of the log of the proliferation ratio on time of culture cv. Pepinex on 0.4 μ M BA
Explant : node 2

```
DATA> end
MTB > save 'T18'
MTB > plot c1 c2
```



$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
C1 = - 0.0210 + 0.0733 Time
```

N_t/N_0

COLUMN	COEFFICIENT	ST. DEV. OF COEF.	T-RATIO = COEF/S.D.
	-0.02100	0.02692	-0.78
Time	0.0732857	0.0006851	106.97

```
S = 0.03217

R-SQUARED =100.0 PERCENT
R-SQUARED =100.0 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE
```

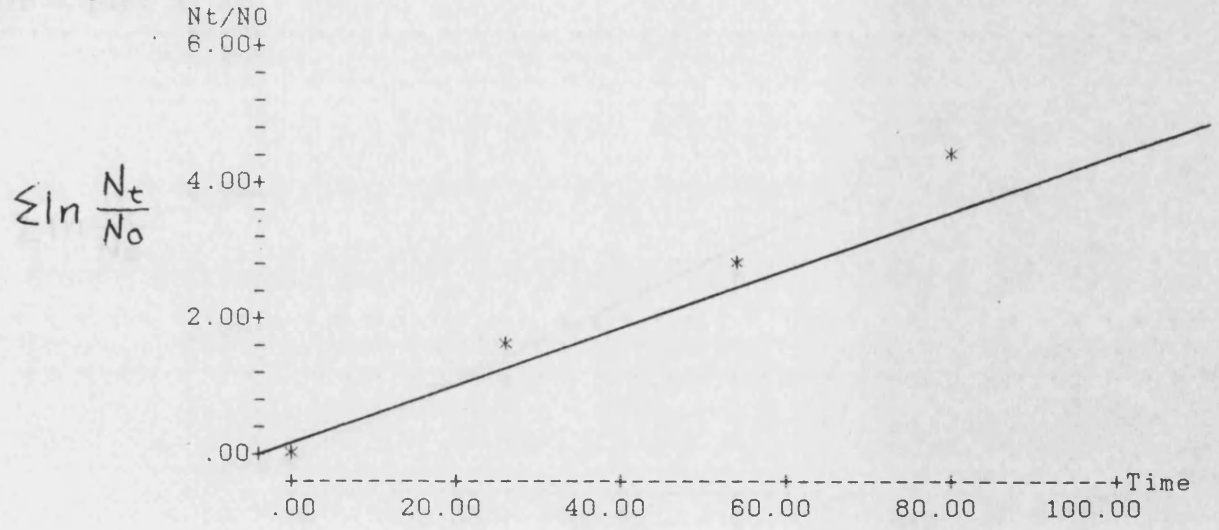
DUE TO	DF	SS	MS=SS/DF
REGRESSION	1	11.843	11.843
RESIDUAL	2	0.002	0.001
TOTAL	3	11.845	

```
DURBIN-WATSON STATISTIC = 2.03

MTB >
```

log of the

Fig 28. Regression analysis of the proliferation ratio on time of culture cv. Telegraph on 0.4μ M BA Explant: shoot tip

```
MTB > save'tr4'
MTB > plot c1 c2
```



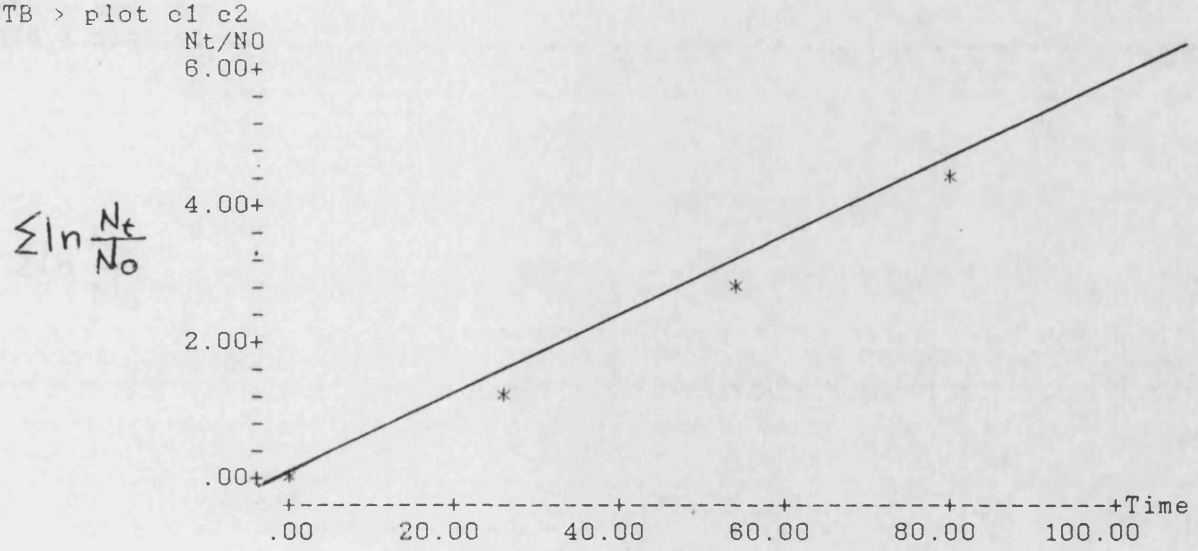
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.0028 + 0.0573 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
              -0.00284        0.02422         -0.12
Time         0.0573042      0.0004873        117.60

S = 0.02909

R-SQUARED =100.0 PERCENT
R-SQUARED =100.0 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       11.706           11.706
RESIDUAL      2        0.002            0.001
TOTAL         3       11.707


DURBIN-WATSON STATISTIC = 3.02

MTB >
```

log of the

Fig 29. Regression analysis of the proliferation ratio on time of culture cv. Telegraph on 0.4 μ M BA Explant : node 1

```
DATA> end
MTB > save'tr5'
MTB > plot c1 c2
```



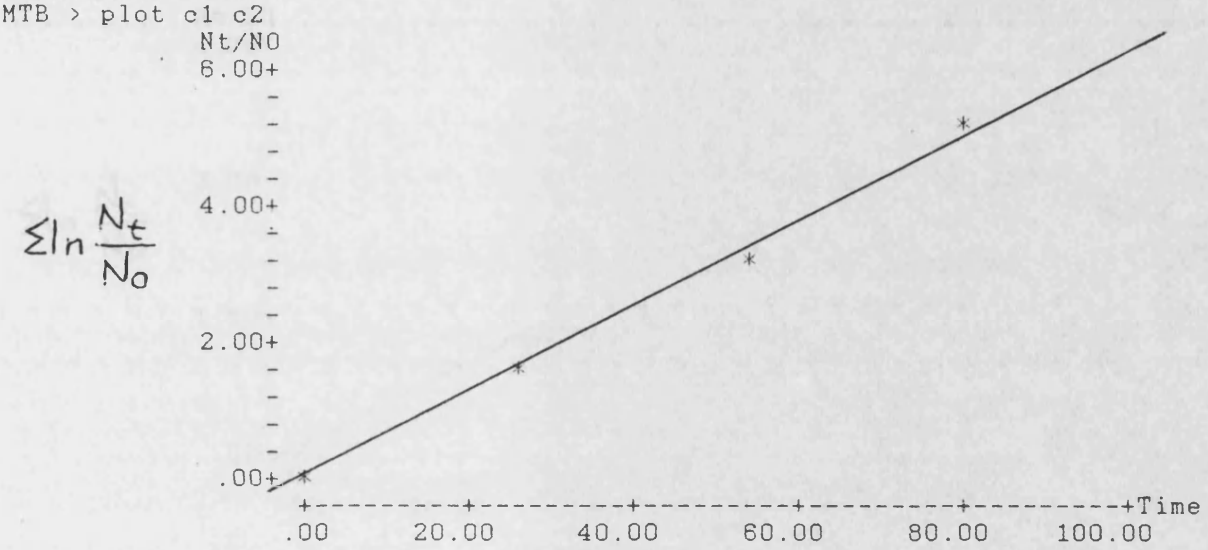
$\Sigma \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.0548 + 0.0562 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.05476          0.05900         -0.93
Time          0.056158         0.001187         47.32

S = 0.07086

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       11.242           11.242
RESIDUAL      2        0.010            0.005
TOTAL         3       11.252


DURBIN-WATSON STATISTIC = 2.09

MTB >
```

log of the

Fig 30. Regression analysis of the proliferation ratio on time of culture cv. Telegraph on 0.4 μ M BA Explant : node 2

```
DATA> end
MTB > save'tr6'
MTB > plot c1 c2
```



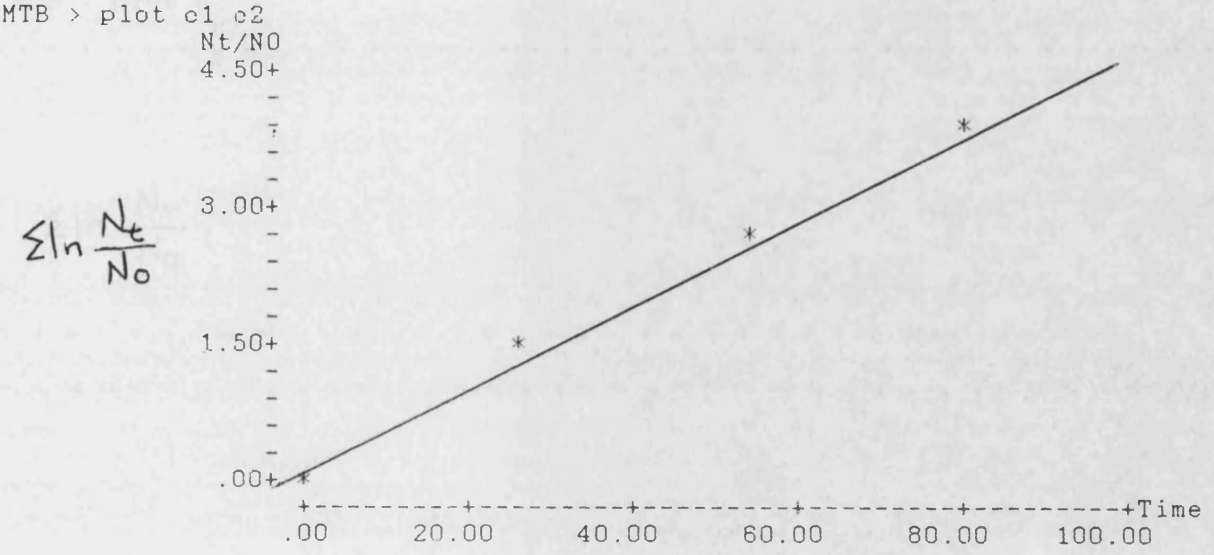
$\Sigma \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.098 + 0.0644 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT        OF COEF.     COEF/S.D.
              -0.0978           0.1135         -0.86
Time         0.064409         0.002283         28.21

S = 0.1363

R-SQUARED = 99.7 PERCENT
R-SQUARED = 99.6 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       14.789           14.789
RESIDUAL      2        0.037            0.019
TOTAL         3       14.826


DURBIN-WATSON STATISTIC = 2.00

MTB >
```

log of the

Fig 31. Regression analysis of the proliferation ratio on time of culture cv. Rebelia on 0.3 μM NAA
Explants: shoot tip

```
DATA> end
MTB > save'tr7'
MTB > plot c1 c2
```

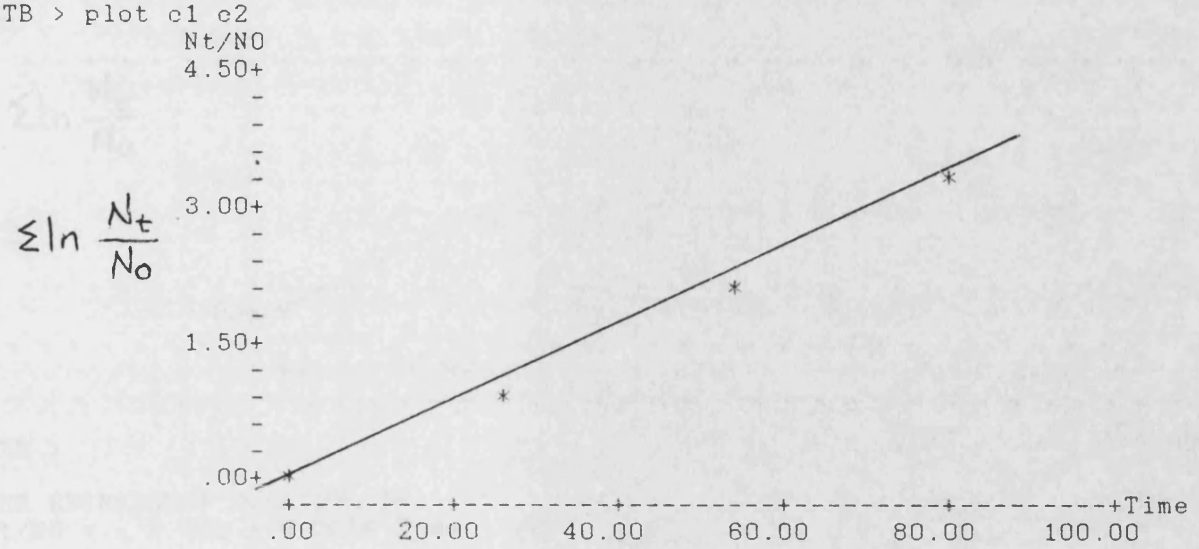


$\Sigma \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = 0.086 + 0.0476 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
                 0.0862           0.1137          0.76
Time          0.047579         0.002288         20.80

S = 0.1366

R-SQUARED = 99.5 PERCENT
R-SQUARED = 99.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       8.0698           8.0698
RESIDUAL      2       0.0373           0.0187
TOTAL         3       8.1071


DURBIN-WATSON STATISTIC = 2.06

MTB >
```

Fig 32. Regression analysis of the log of the proliferation ratio on time of culture cv. Rebelia on 0.3 µM NAA
Explant : node 1

```
DATA> end
MTB > save'tr8'
MTB > plot c1 c2
```



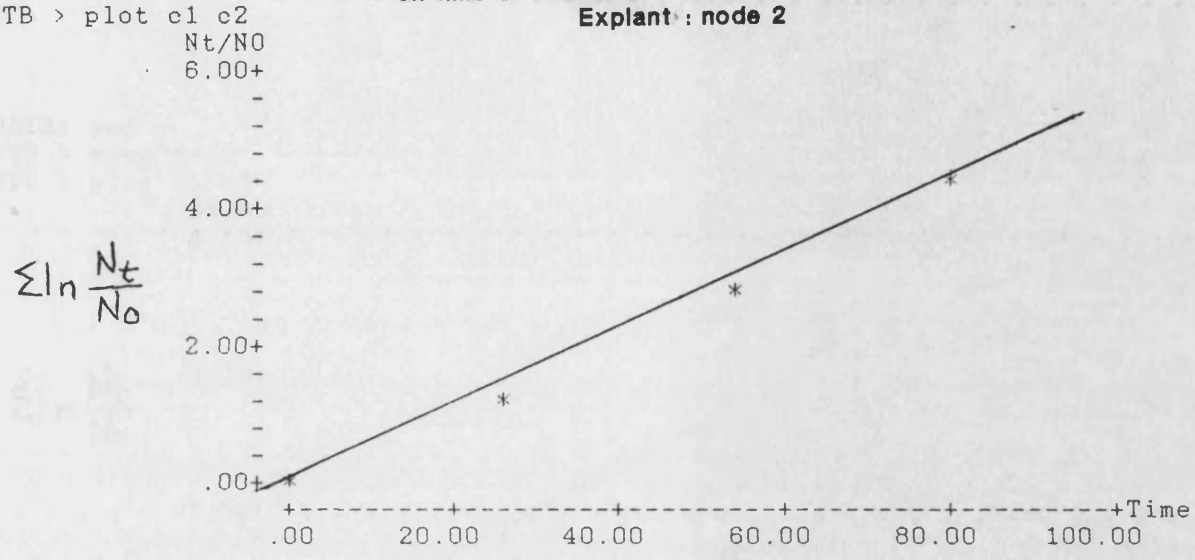
$\Sigma \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.099 + 0.0432 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT        OF COEF.     COEF/S.D.
                -0.0991          0.1114         -0.89
Time          0.043248        0.002240          19.30

S = 0.1338

R-SQUARED = 99.5 PERCENT
R-SQUARED = 99.2 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       6.6676           6.6676
RESIDUAL      2       0.0358           0.0179
TOTAL         3       6.7034


DURBIN-WATSON STATISTIC = 2.01

MTB >
```

log of the

Fig 33. Regression analysis of the proliferation ratio on time of culture cv. Rebelia on 0.3 μM NAA Explant : node 2

```
DATA> end
MTB > save'tr9'
MTB > plot c1 c2
```



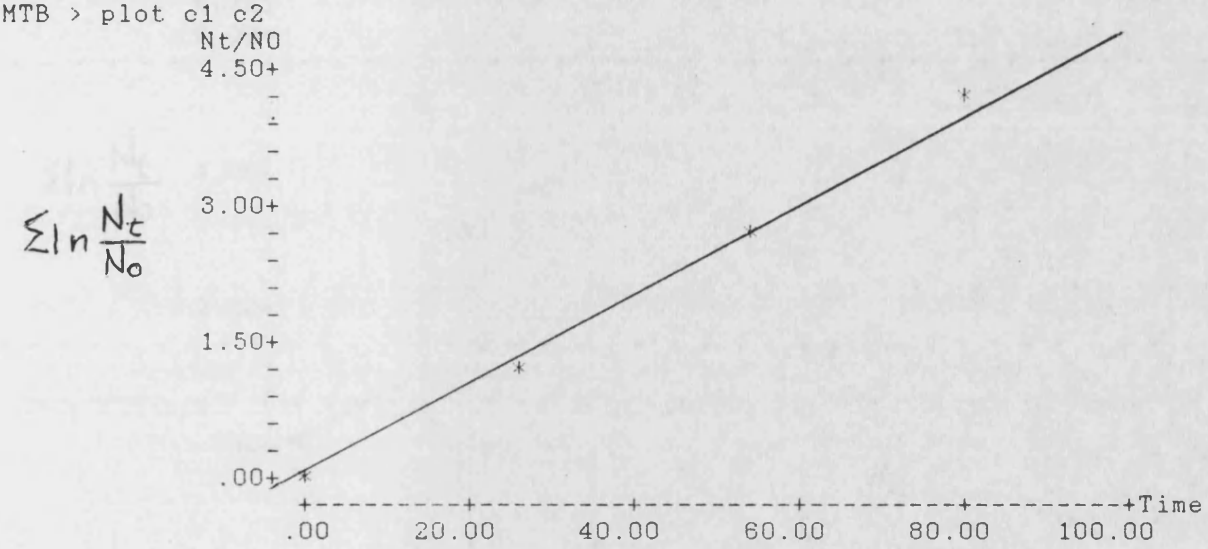
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.126 + 0.0554 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
               -0.1258           0.1345         -0.94
Time          0.055428         0.002705         20.49

S = 0.1615

R-SQUARED = 99.5 PERCENT
R-SQUARED = 99.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF            SS         MS=SS/DF
REGRESSION    1        10.952           10.952
RESIDUAL      2         0.052            0.026
TOTAL         3        11.004


DURBIN-WATSON STATISTIC = 2.13

MTB >
```

log of the

Fig 34. Regression analysis of the proliferation ratio on time of culture cv. Rebella on 0.4 μ M BA
Explant : shoot tip

```
DATA> end
MTB > save'tr10'
MTB > plot c1 c2
```

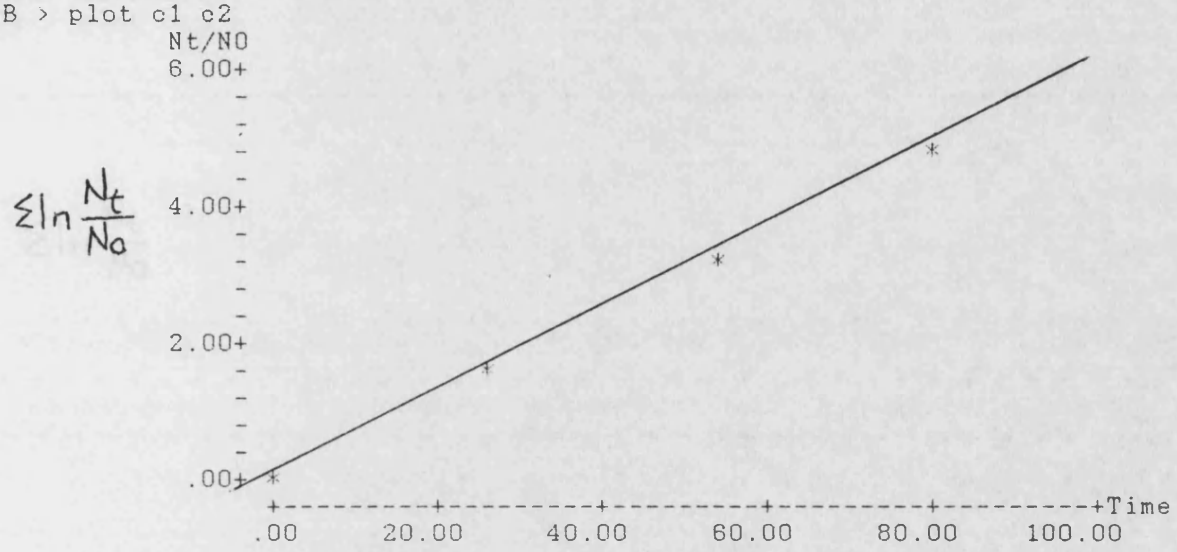


$\sum \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.0821 + 0.0528 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.08208          0.08808         -0.93
Time           0.052757        0.001772         29.78

S = 0.1058

R-SQUARED = 99.8 PERCENT
R-SQUARED = 99.7 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        9.9217          9.9217
RESIDUAL      2        0.0224          0.0112
TOTAL         3        9.9441


DURBIN-WATSON STATISTIC = 2.11

MTB >
```

Fig 35. Regression analysis of the log of the proliferation ratio on time of culture cv. Rebelia on 0.4 μ M BA
Explant : node 1

```
DATA> end
MTB > save 'tr11'
MTB > plot c1 c2
```

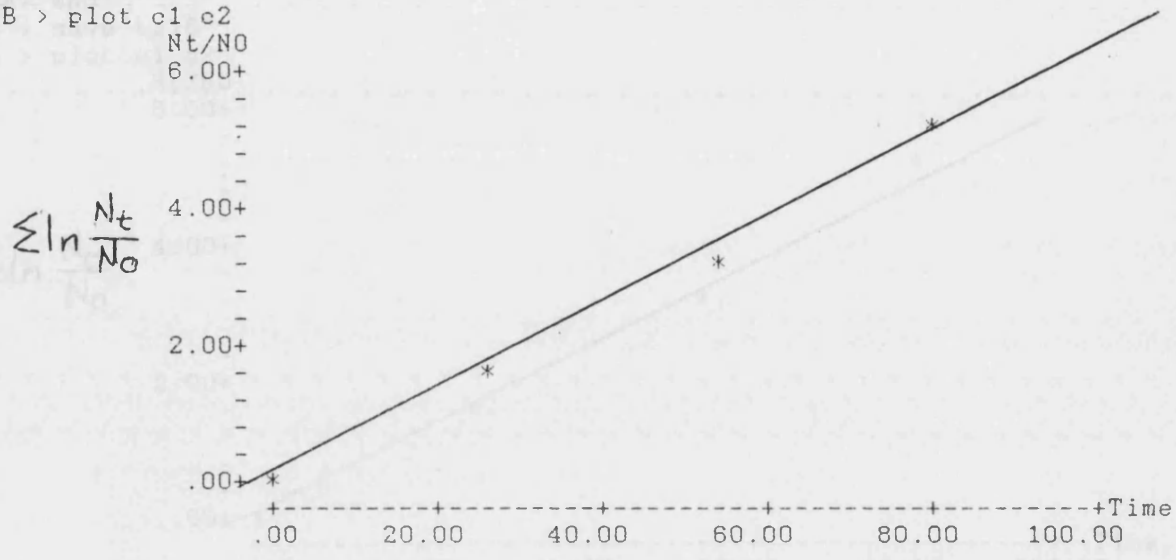



```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.101 + 0.0615 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
                -0.1007           0.1239         -0.81
Time          0.061526         0.002493         24.68

S = 0.1488

R-SQUARED = 99.7 PERCENT
R-SQUARED = 99.5 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS       MS=SS/DF
REGRESSION    1       13.494         13.494
RESIDUAL      2        0.044          0.022
TOTAL         3       13.538


DURBIN-WATSON STATISTIC = 2.02

MTB >
```

log of the

Fig 36. Regression analysis of the proliferation ratio on time of culture cv. Rebella on 0.4 μ M BA
Explant : node 2

```
DATA> 0  26 53 80
DATA> save'tr12'
MTB > plot c1 c2
```



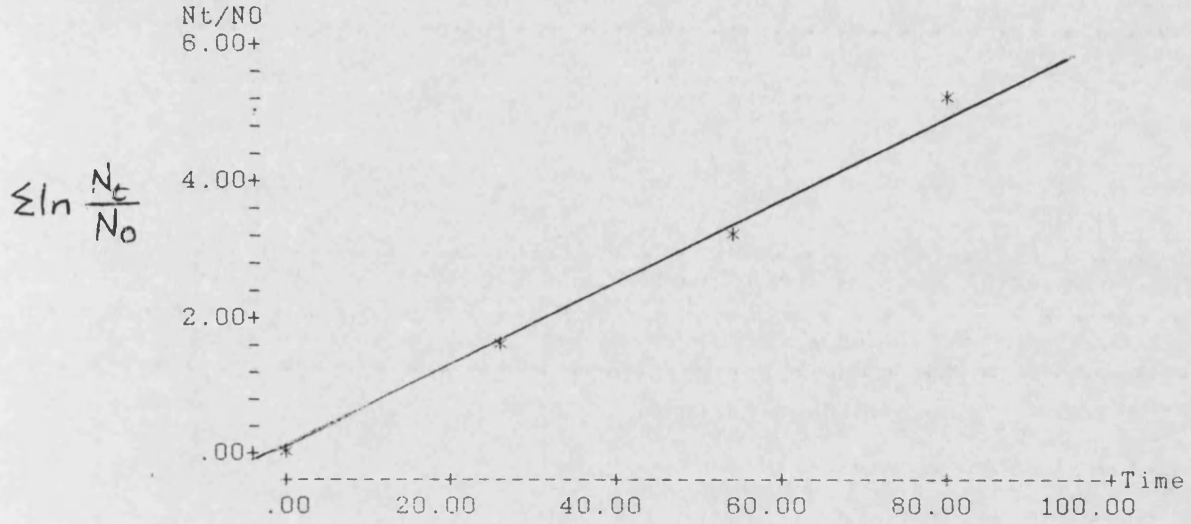
$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.0720 + 0.0628 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.07197          0.07951         -0.91
Time           0.062754        0.001599         39.24

S = 0.09549

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.8 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        14.038          14.038
RESIDUAL      2         0.018           0.009
TOTAL         3        14.056


DURBIN-WATSON STATISTIC = 2.03

MTB >
```

log of the

Fig 37. Regression analysis of the proliferation ratio on time of culture cv. Pepinex on 0.4 μ M BA Explant. : shoot tip

```
DATA> end
MTB > save'tr16'
MTB > plot c1 c2
```

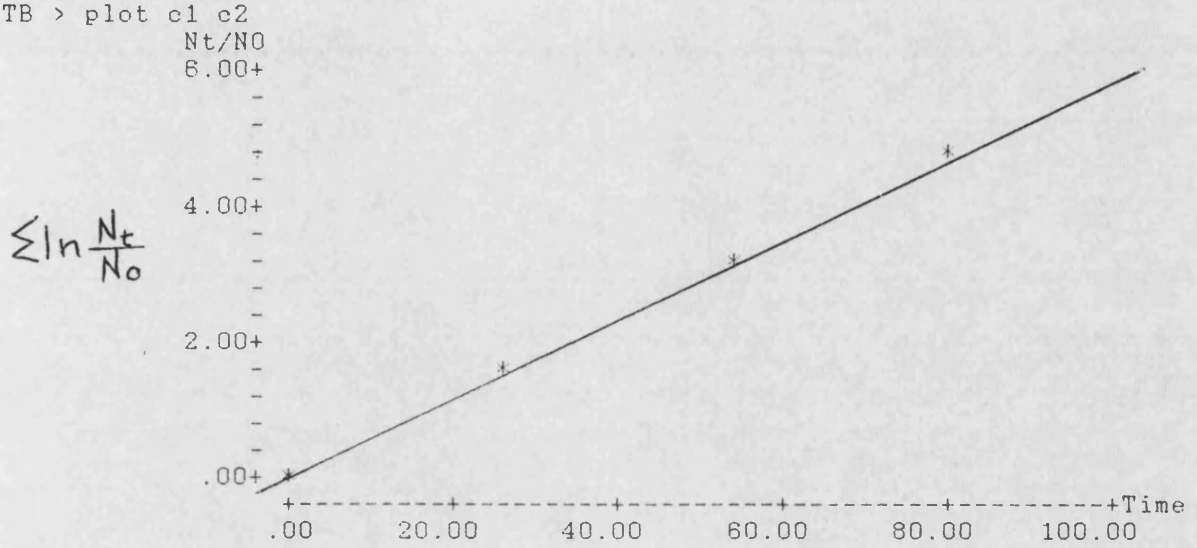



```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.0619 + 0.0628 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.06186          0.06788         -0.91
Time          0.062751         0.001365         45.96

S = 0.08152

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       14.037           14.037
RESIDUAL      2        0.013            0.007
TOTAL         3       14.050


DURBIN-WATSON STATISTIC = 2.04

MTB >
```

log of the

Fig 38. Regression analysis of the proliferation ratio on time of culture cv. Pepinex on 0.4 μ M BA Explant : node 1

```
DATA> end
MTB > save'tr17'
MTB > plot c1 c2
```



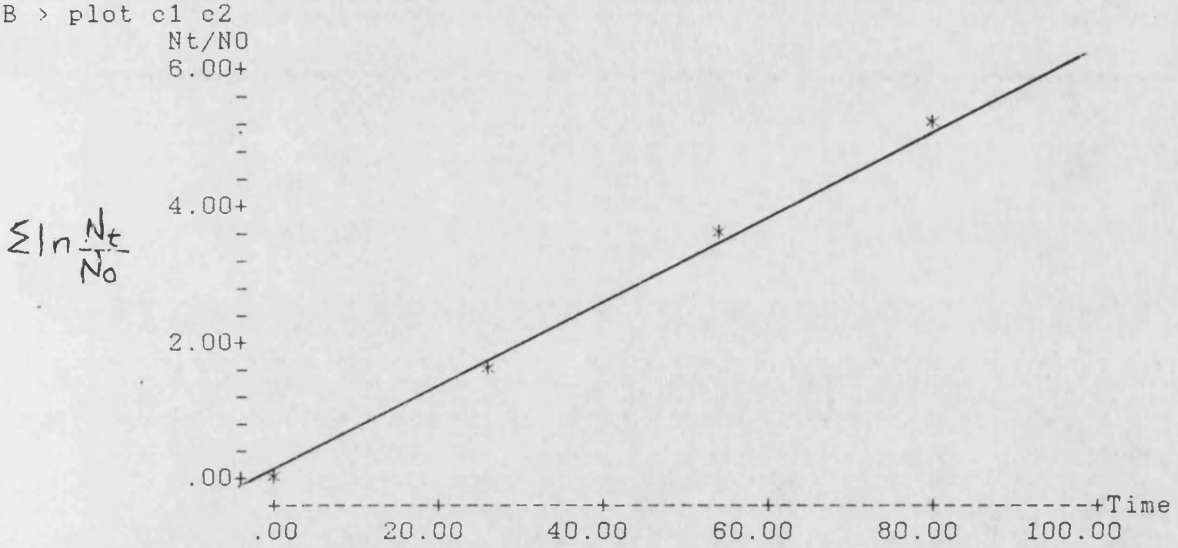
$\Sigma \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.0357 + 0.0591 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
              -0.03567         0.03912        -0.91
Time         0.0590736       0.0007870        75.06

S = 0.04699

R-SQUARED =100.0 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       12.440           12.440
RESIDUAL      2        0.004            0.002
TOTAL         3       12.444


DURBIN-WATSON STATISTIC = 2.04

MTB >
```

log of the

Fig 39. Regression analysis of the proliferation ratio on time of culture cv. Pepinex on 0.4 μ M BA
Explant : node 2

```
DATA> end
MTB > save'tr18'
MTB > plot c1 c2
```

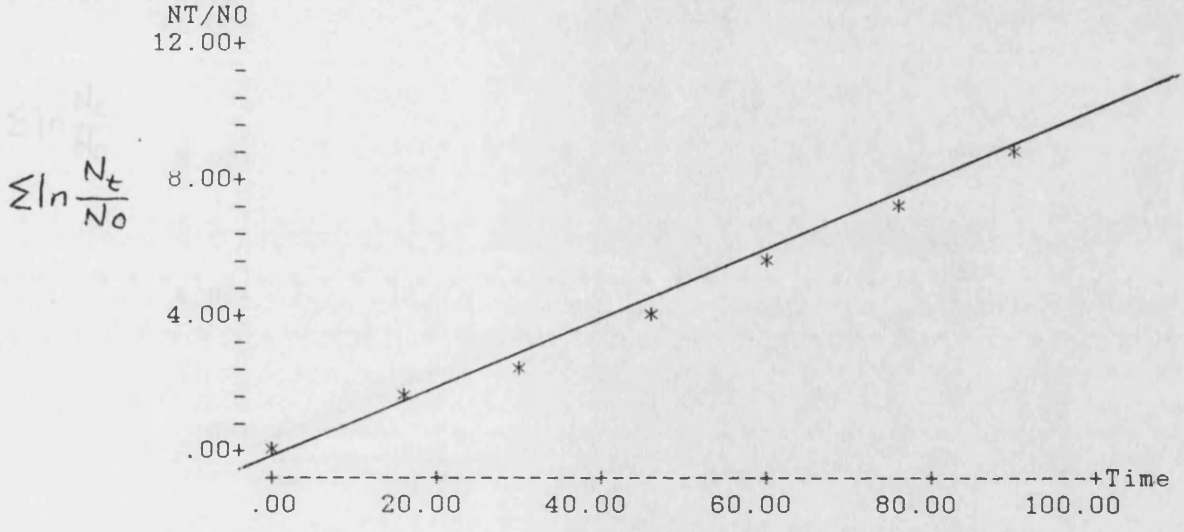


$\Sigma \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.0466 + 0.0665 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
             -0.04658           0.05157        -0.90
Time          0.066455         0.001037        64.06

S = 0.06194

R-SQUARED =100.0 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1        15.743          15.743
RESIDUAL      2         0.008           0.004
TOTAL         3        15.750


DURBIN-WATSON STATISTIC = 2.03

MTB >
```

Fig 45. Regression analysis 15 day intervals on cv. Telegraph at 30°C on 0.35 μ M BA
Explants: shoot tip

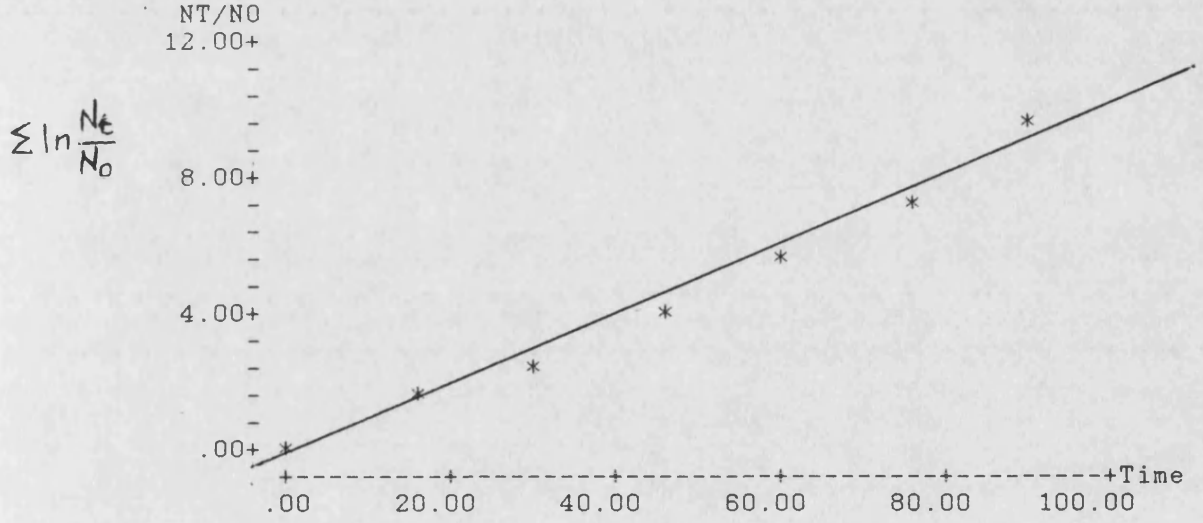
```
MTB > retr 'T-Fifte'
MTB > plot c1 c2
```




```
MTB >

THE REGRESSION EQUATION IS
NT/NO = - 0.257 + 0.0996 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              -0.2575            0.1310         -1.97
Time         0.099595          0.002422         41.13

S = 0.1922

R-SQUARED = 99.7 PERCENT
R-SQUARED = 99.6 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       62.491           62.491
RESIDUAL      5        0.185            0.037
TOTAL         6       62.676


DURBIN-WATSON STATISTIC =   .83

MTB >
```

Fig 46. Regression analysis 15 day intervals on cv. Rebelia at 30°C on 0.35 μM BA Explants: shoot tip

```
DATA> end
MTB > save'R-Fifte'
MTB > plot c1 c2
```

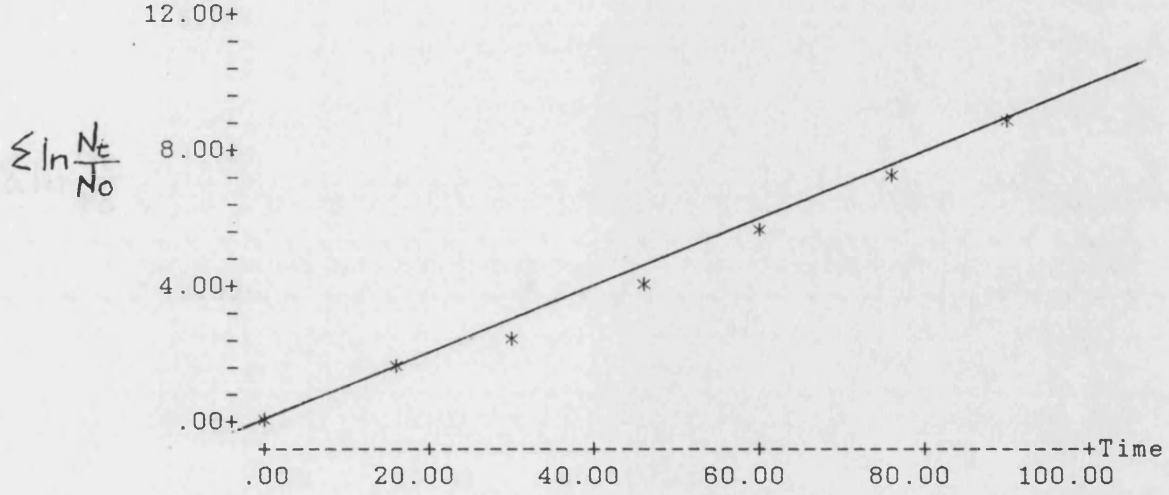


$\Sigma \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
NT/NO = - 0.255 + 0.105 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT        OF COEF.     COEF/S.D.
              -0.2546           0.1454         -1.75
Time         0.104738         0.002688          38.96

S = 0.2134

R-SQUARED = 99.7 PERCENT
R-SQUARED = 99.6 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       69.111           69.111
RESIDUAL      5        0.228            0.046
TOTAL         6       69.339


DURBIN-WATSON STATISTIC =  .84

MTB >
```

Fig 47. Regression analysis 15 day intervals on cv. Pepinex at 30°C on 0.35 μ M BA
Explants: shoot tip

```
DATA> end
MTB > save'P-fifte'
MTB > plot c1 c2
```

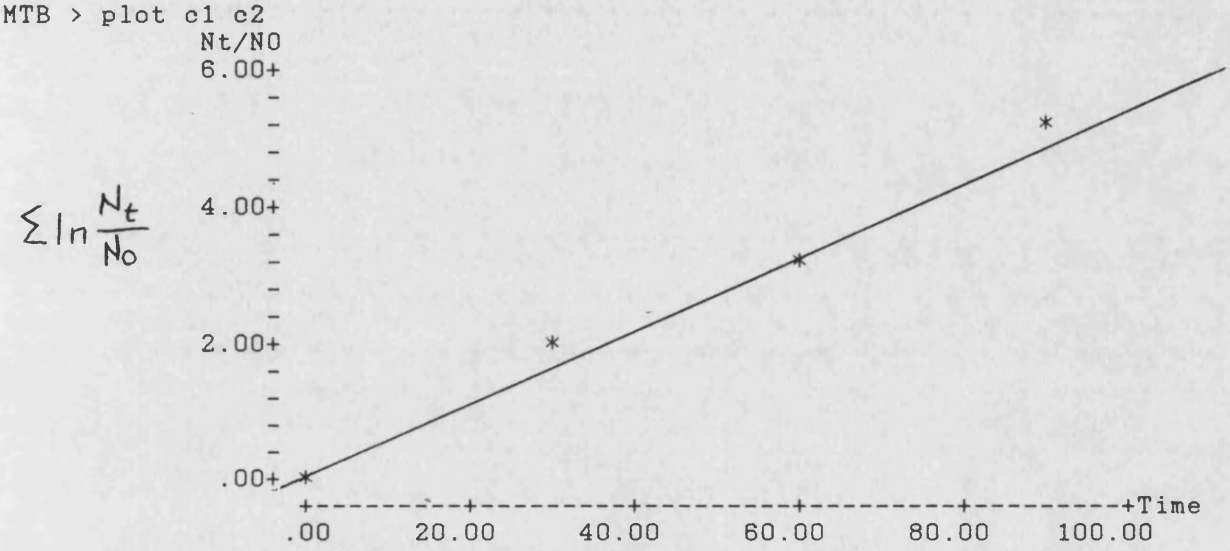


$\sum \ln \frac{N_t}{N_o}$

```
MTB >

THE REGRESSION EQUATION IS
NT/NO = - 0.165 + 0.0979 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
              -0.16500        0.07694        -2.14
Time          0.097857       0.001423        68.78

S = 0.1129

R-SQUARED = 99.9 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS         MS=SS/DF
REGRESSION    1       60.329           60.329
RESIDUAL      5        0.064            0.013
TOTAL         6       60.393


DURBIN-WATSON STATISTIC = 1.37

MTB >
```

Fig 48. Regression analysis 30 day intervals on cv. Telegraph at 30°C on 0.35 μ M BA Explant: shoot tip

```
y
MTB > retr 'Tel-30'
MTB > plot c1 c2
```

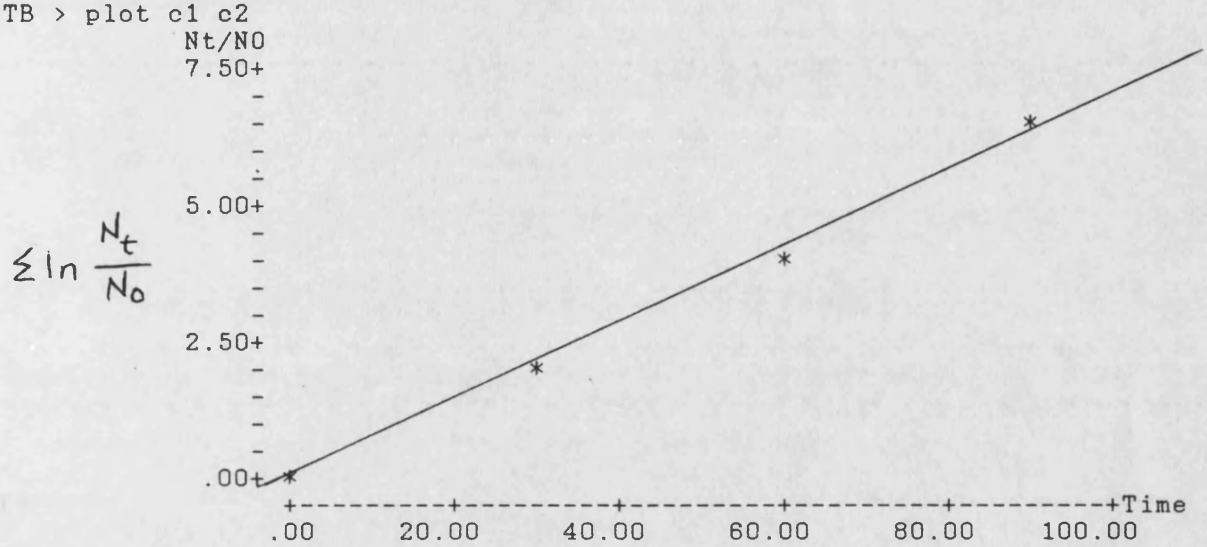


$\sum \ln \frac{N_t}{N_0}$

```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = 0.063 + 0.0538 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
              0.0630            0.2278          0.28
Time          0.053767          0.004058        13.25

S = 0.2722

R-SQUARED = 98.9 PERCENT
R-SQUARED = 98.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS          MS=SS/DF
REGRESSION    1        13.009          13.009
RESIDUAL      2         0.148           0.074
TOTAL         3        13.157


DURBIN-WATSON STATISTIC = 3.38

MTB >
```

Fig 49. Regression analysis 30 day intervals on cv. Rebella at 30°C on 0.35 μM BA
Explant: shoot tip

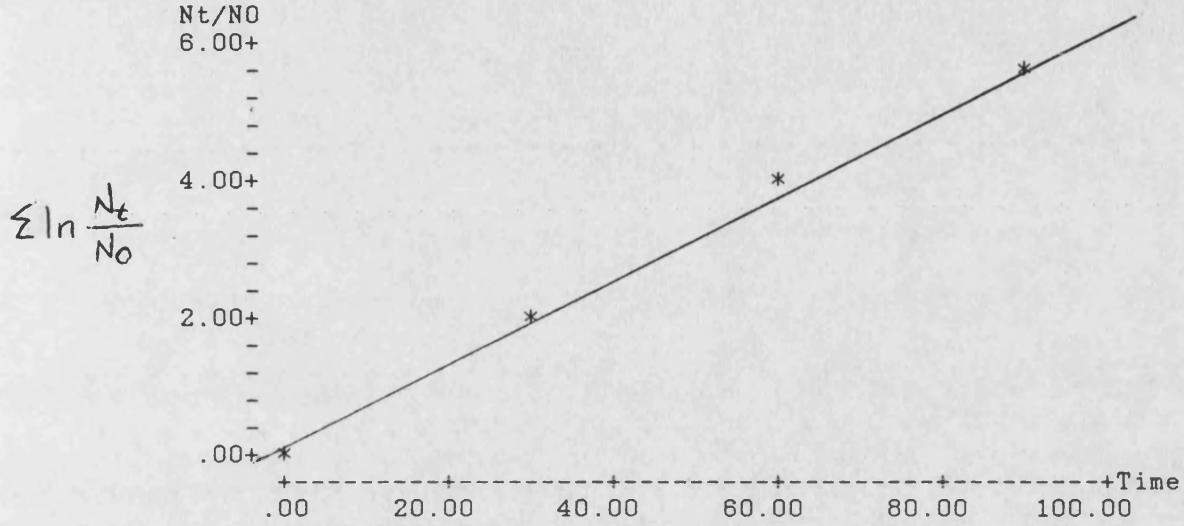
```
DATA> end
MTB > save'Reb-30'
MTB > plot c1 c2
```




```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = - 0.0560 + 0.0696 Time

                              ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT       OF COEF.     COEF/S.D.
             -0.05600         0.06054         -0.93
Time          0.069633       0.001079         64.56

S = 0.07235

R-SQUARED =100.0 PERCENT
R-SQUARED = 99.9 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO     DF           SS       MS=SS/DF
REGRESSION   1       21.820         21.820
RESIDUAL     2        0.010          0.005
TOTAL        3       21.830


DURBIN-WATSON STATISTIC = 2.19

MTB >
```

Fig 50. Regression analysis 30 day intervals on cv. Pepinex at 30°C on 0.35 μM BA
Explant: shoot tip

```
DATA> end
MTB > save'Pep-30'
MTB > plot c1 c2
```




```
MTB >

THE REGRESSION EQUATION IS
Nt/NO = 0.042 + 0.0627 Time

                                ST. DEV.     T-RATIO =
COLUMN      COEFFICIENT         OF COEF.     COEF/S.D.
                 0.0420           0.1381          0.30
Time          0.062733         0.002460         25.50

S = 0.1650

R-SQUARED = 99.7 PERCENT
R-SQUARED = 99.5 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS        MS=SS/DF
REGRESSION    1       17.710          17.710
RESIDUAL      2        0.054           0.027
TOTAL         3       17.764


DURBIN-WATSON STATISTIC = 2.74

MTB >
```